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CHARACTERIZATION OF THE FECAL INDICATOR BACTERIAL FLORA OF SANITARY SEWAGE WITH APPLICATION TO IDENTIFYING THE PRESENCE OF SANITARY WASTE IN STORM SEWERS

R. A. C. PROJECT NO. 247PL

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PUBLICATIONS RELATED TO THIS PROJECT

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ABSTRACT

Storm sewers are designed to channel untreated storm water into surface waters. An unusually high dry weather flow of storm sewage along with high fecal coliform counts indicate that there may be potentially hazardous sanitary connections in the storm sewer line. The objective of this study was to assess the use of bacterial indicators to trace illegal sanitary connections to storm sewers. The indicators selected for study were Escherichia coli, fecal coliforms, fecal streptococci, enterococci, Pseudomonas aeruginosa, Clostridium perfringens, and Bifidobacterium sp. The organisms were collected during periods of wet and dry weather from October, 1986 to August, 1988. The sampling sites A, B and C in the Mount Steven Trunk storm sewer line were selected because this area was designated a high priority sewer by the Ministry of the Environment. Non-priority sites, labelled X, Y and Z in the Mount Steven Trunk storm sewer branch lines were sampled for comparison. For wet weather, storm water run-off was collected at the X, Y and Z sites. Samples were also obtained from a sanitary sewer in close proximity to the priority storm sewer sampling points; these sites were labelled D, E and F.

Biochemical testing, serotyping, and/or genotyping were used to further characterize more than 4,000 fecal streptococcus, Pseudomonas aeruginosa, and Bifidobacterium spp. isolates. Speciation of the fecal streptococci showed that Streptococcus faecalis subsp. faecalis was more predominant in sanitary and high priority sewers than in surface runoff and non-priority sewers. Streptococcus faecium subsp. casseliflavus, on the other hand, was found primarily in surface water runoff and non-priority storm sewers. Litmus milk reactions among the S. faecalis isolates generally did not assist in source tracing. DNA sequence studies of the fecal streptococci, using Restriction

Endonuclease Analysis (REA), produced many different restriction patterns and it was difficult to establish any relationship between the isolates. Although serotyping of Pseudomonas aeruginosa was found to produce nonspecific results, genotyping did provide a precise method of fingerprinting the organisms. Attempts to genotype the bifidobacteria were unsuccessful; however, members of the genus show promise as pollution indicators because they were found in high concentrations in human feces and sanitary sewage. Further work to determine the natural sources of Bifidobacterium species should be carried out.

CONCLUSIONS

The results of the study showed that the levels of bacterial indicators, particularly fecal coliforms, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bifidobacterium* spp., were high in the high priority storm sewer line at or near sampling points A, B and Y. The data suggest that there is an impact near site A in the storm sewer line that may be due to human fecal pollution.

Characterization of the indicator bacteria by means of biochemical testing, serotyping and genotyping produced notable results. For example, speciation of the fecal streptococci was shown to be a useful means of identifying sewer or storm sewer water content. The *Streptococcus* species *S. faecium* tended to be equally represented in all types of samples. *Streptococcus faecalis* subsp. *faecalis*, on the other hand, was found with greater frequency in sanitary and priority storm sewers than in surface runoff and non-priority sewers. In contrast, *S. faecium* subsp. *casseliflavus* was almost nonexistent in sanitary sewage but was the predominant enterococcus in non-priority storm sewer water.

Mundt's 1973 *S. faecalis* studies showed that isolates from non-human sources produced proteinization reactions in litmus milk whereas the isolates from human feces yielded acid curds. Such a trend was not observed among the *S. faecalis* species isolated in this investigation and therefore the litmus milk reactions do not appear to be a useful criterion in source tracing. As well, genotyping of the faecal streptococcal isolates from the sanitary sewer, priority and non-priority storm sewers and the surface runoff did not produce conclusive results. A total of 64 different restriction patterns were identified among the 192 streptococcal isolates examined and the patterns did not show any distinctive trends.

Fecal coliform to fecal streptococcus ratios have been proposed as a method of estimating whether the source of pollution was from a human or nonhuman source. The FC/FS ratios were calculated during the course of this study but the results were inconclusive. Ratios greater than 4.0, suggesting a human source, were consistently found in the sanitary sewage but the ratios in the remainder of the samples varied too widely for any assumptions to be made.

Pseudomonas aeruginosa counts were highest in sanitary and priority storm sewage; they reached their lowest levels in non-priority storm sewage. Serotype 0:6 was the predominant type in all samples collected. Also evident were serotypes 0:1, 0:10, 0:11, 0:4, 0:3 and 0:2. There was a tendency for serotype 0:10 to be found primarily in sanitary sewage, but otherwise Pseudomonas serotyping did not appear to be applicable to source differentiation. Genotyping P. aeruginosa organisms, on the other hand, did produce a more precise method of fingerprinting the isolates. For example, the eight different genotypes that were obtained from the sanitary and high priority storm sewer cultures were not found in the non-priority sewer and surface water runoff isolates. Also, similar REA patterns were observed in the P. aeruginosa isolates from storm water runoff and the corresponding storm sewer site.

Studies in our laboratory have shown that Bifidobacterium spp. are present in human feces in concentrations of approximately 1 x 10^8 per gram. The results of this investigation showed that bifidobacteria counts were in the 10^4 to 10^5 per 100mL range in sanitary and high priority storm sewage and decreased by two orders of magnitude in non-priority storm sewage. Mara and Oragui (1983) have suggested that sorbital fermenting bifidobacteria (i.e B. adolescentis and B. breve) could be used as indicators of human fecal pollution. However, we would suggest caution in this approach because we have been able to isolate B. adolescentis from dog feces and B. breve, B. minimum and B. thermophilum from chicken fecal samples. It is interesting that the nonhuman B. thermophilum strain was also isolated from the non-priority storm sewer at site Z.

Although three different restriction enzymes were used to digest whole cell DNA from the bifidobacteria not enough DNA was recovered and the genotyping experiments were unsuccessful. This aspect of the investigation merits further study.

RECOMMENDATIONS

1. This project showed that source determination studies can be conducted most effectively during periods of dry weather when there is a substantial flow in the storm sewer line. During storm events it is difficult to trace the source of contamination because the bacterial indicators come from a variety of sources. It is therefore recommended that source tracing be done only under dry weather conditions.
2. The results of this investigation suggest that because S. faecium subsp. casseliflavus was isolated primarily from surface water runoff and non-priority storm sewers it may come solely from nonhuman sources. This concept is supported by the fact that we have been unable to isolate the organism from a limited number of human fecal specimens. We propose that further studies be carried out to determine the source of this organism. Also, that field studies be done to assess the applicability of a E. coli to S. casseliflavus or a Bifidobacterium to S. casseliflavus ratio.
3. It was apparent from this project that the serotyping of P. aeruginosa

isolates did not aid in the tracing of illegal sanitary connections in storm sewers. It is suggested that in future studies serotyping be combined with phage typing or genotyping to fingerprint the Pseudomonas organisms. A probe, developed from either total chromosomal DNA or a specific fragment common to human strains, might allow us to hybridize against all other strains and differentiate between human and nonhuman fecal material.

4. The project results suggest that bifidobacteria show promise as pollution indicators. Additional work should be done to determine the natural habitat of the individual Bifidobacterium species. In addition, further attempts could be made to genotype the organisms.

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ABBREVIATIONS

REA - Restriction Endonuclease Analysis

FC - fecal coliforms

FS - fecal streptococcus

P. - Pseudomonas

B. - Bifidobacterium

S. - Streptococcus

E. - Escherichia

L - litre

IG - indoxyl-β-D-glucoside

SDS - sodium dodecyl sulphate

EDTA - ethylenediaminetetraacetic acid

TAE - Tris base, 1.0 sodium acetate, 0.1 M disodium EDTA

CFU - colony forming units

Var - variety

ATCC - American Type Culture Collection

E - exponential

INTRODUCTION

Storm sewers were designed to channel storm water from urban areas into surface waters primarily to avoid the flooding that would occur as a consequence of the blocking of natural flow patterns that existed before urbanization. The content of storm sewers should be similar to direct runoff and the flow restricted primarily to storm events. A small amount of dry weather flow is not unusual in storm sewer lines due to ground water intrusion and human activities such as the watering of lawns. However, significant dry weather flow coupled with high fecal coliform counts may indicate that illegal sanitary connections are present somewhere in the storm sewer line. This situation presents a health hazard since storm sewage is not normally treated prior to entering receiving waters such as Lake Ontario beach areas or the Humber and Don Rivers which flow into Lake Ontario near beaches.

Reports of the Toronto Area Watershed Management Study (TAWMS) in 1983 and 1984 have identified contaminated storm sewer outfalls as a major source of bacterial pollution loadings to the lower Humber River watershed (Gartner Lee and Assoc. 1983; Weatherbee and Novak, 1984). The 1983 outfall inventory study of the lower Humber River basin identified eighty-four dry weather outfalls that were considered sufficiently active to warrant intensive sampling and testing (Gartner Lee and Assoc. 1983). A similar study initiated in the Don River watershed indicated that 16% of all dry weather outfalls exceeded the recommended guidelines for fecal coliform densities (TAWMS Tech. Rep. No. 11, 1986).

The Ministry of the Environment has designated storm sewer outfalls discharging more than 1 L/sec. during dry weather periods and exhibiting fecal coliform densities of greater than 10,000 FC/100 mL as being high priority and have called upon municipal agencies to identify and eliminate the source of the fecal pollution in these sewage lines. Follow up action has been initiated on identified outfalls in the Humber River watershed and already several illegal cross-connections between sanitary and storm sewers have been located and corrected (Weatherbee and Novak, 1984). Despite some successful attempts, compliance with the Ministry's directive can be difficult at times, due to the fact that methods for source determination are under-developed. Escherichia coli which is an excellent indicator of fecal contamination does not lend itself to source determination due to its wide-spread occurrence in all fecal material. Older methods such as the ratio of fecal coliforms to fecal streptococci (FC/FS) have been used. However, the ratios proposed by Geldreich in 1969 can no longer be considered valid. A major reason for this is that bacterial isolation and enumeration methods currently used in Ontario differ significantly from those used to develop the FC/FS ratio. As well, it has been shown that certain animal hosts; i.e. dogs, gulls and pigeons exhibit similar ratios to that of humans (Seyfried, Harris and Young, 1986 unpublished data) and that the ratios obtained from polluted waters change over time as a result of environmental stress (Seyfried, Harris and Young, 1986 unpublished data; Feachem, 1975).

Recently, newer methods of source determination have been proposed. These methods entail the use of a variety of indicator organisms as described below.

Rationale for the Use of Selected Indicator Organisms

The levels of pathogens in polluted water and animal feces are highly variable since the densities reflect the intestinal diseases that are prevalent in human or other animal populations at a given time. As a result, monitoring of water for possible detection of waterborne pathogens requires a variety of complex, time-consuming, and often insensitive procedures. The use of a bacterial indicator system that will detect and measure fecal pollution from all warm-blooded animals is a more realistic approach.

Fecal Coliforms

The fecal coliform bacteria do have a direct correlation with fecal contamination from warm-blooded animals. The ability to ferment lactose with gas production at 44.5°C is the principal biochemical characteristic used to identify fecal coliforms. Geldreich (1966) has shown that 96.4% of the coliforms in human feces were positive by this test. Soils that are contaminated with fecal discharges or exposed to polluted water will contain varying levels of fecal coliforms (Van Donsel, Geldreich and Clark, 1967; Geldreich _et al._, 1968). The low levels of coliforms that have been detected on vegetation are derived from animal manure or night-soil used as fertilizers, or by contact with contaminated insects and agricultural pests (Geldreich, Kenner and Kabler, 1964). During periods of rainfall, contamination that might be associated with vegetation could enter surface waters via stormwater drainage (Geldreich _et al._, 1968).

Escherichia coli

In Europe, there is a long standing tradition to distinguish between total coliforms and _Escherichia coli_ because the latter is considered to be more significant (Leclere _et al._, 1977). According to Cabelli (1977), _E. coli_ is the only coliform biotype that is consistently and exclusively associated with fecal wastes of warm-blooded animals. Thus the significance of the presence of E. coli in surface waters is that fecal contamination due to humans or warm-blooded animals has occurred and therefore a potential health hazard risk from microbial or viral enteric pathogens does exist. The levels of E. coli found in various animals, birds, and sewage effluents are given in the table below.

Table 1. Levels of E. coli

(from Jones and White, 1983)

	Fecal production g/d	Average number E. coli/g faeces	Daily load E. coli
Man	150	13 x 10^6	1.9 x 10^9
Cow	23600	0.23 x 10^6	5.4 x 10^9
Hog	2700	3.3 x 10^6	8.9 x 10^9
Sheep	1130	16 x 10^6	18.1 x 10^9
Duck	336	33 x 10^6	11.1 x 10^9
Turkey	448	0.3 x 10^6	0.13 x 10^9
Chicken	182	1.3 x 10^6	0.24 x 10^9
Gull	15.3	131.2 x 10^6	2 x 10^9
	E. coli concentration 100 ml		
Sewage	3.4 x 10^3 - 2.8 x 10^7		
Sewage effluent	1 x 10^3 - 10^7		

E. coli survival

Freshwater	mean T_{90} 62.3 h
Seawater	mean T_{90} 2.3 h

Dufour's (1977) finding that E. coli showed the best relationship to gastrointestinal illness, rather than members of the Klebsiella-Enterobacter-Citrobacter group, is not surprising in view of its position of dominance in the distribution of coliforms found in fecal wastes from humans and other warm-blooded animals.

Fecal Streptococci and Enterococci

Fecal streptococci are defined as those species of streptococci which are recovered from feces in significant quantities (Clausen et al., 1977).

The term enterococci is used to describe those species of fecal streptococci that grow at both 10° and 45° C and in the presence of 40% bile. Growth should occur at a pH of 9.6 and in the presence of 6.5% sodium chloride. Esculin hydrolysis is also positive for these organisms. The group D enterococci include *S. faecalis*, *S. faecium*, *S. durans*, *S. avium*, and related biotypes (Bergey's Manual, 1986).

The occurrence of fecal streptococci in water suggests fecal pollution and their absence indicates little or no warm-blooded animal contamination (Geldreich and Kenner, 1969). There is no indication that they multiply in natural or fecally polluted waters or soils (Clausen et al., 1977). Cabelli (1977) has suggested that the enterococcus group most closely meets the characteristics of an ideal indicator, particularly because it survives better than *E. coli* in the aquatic environment.

A study conducted at the University of Toronto (Seyfried, Harris and Young, 1986 unpublished) found that differences in the relative proportions and biotypes of group D streptococci existed between human and animal hosts. These findings are also supported by previous workers (Wheater, et al. 1979, Kenner

1978; and Geldreich 1976). Hill et al. (1971) found that human subjects fed on a mainly mixed western diet carried a higher percentage of S. faecalis biotypes. Mundt (1982) also found S. faecalis to be the predominant species of group D streptococci in humans and was able to demonstrate marked differences in the litmus milk reactions of S. faecalis from humans, animals and plant material (Mundt, 1973).

Fecal Coliform to Fecal Streptococci Ratios

The FC/FS ratio has been proposed as a means of estimating whether the pollution originated from a human or a nonhuman source (Geldreich, 1966; Geldreich and Kenner, 1969, Geldreich et al. 1968). Geldreich suggests that, "Fecal coliform bacteria are more numerous than fecal streptococci in domestic sewage, with a fecal coliform to fecal streptococcus ratio always greater than 4.0. As might be expected, similar ratios are common to the feces of man. Conversely, fecal streptococci are more numerous than fecal coliforms in the feces of farm animals, cats, dogs and rodents. In feces from these animals, the fecal coliform (FC) to fecal streptococcus (FS) ratios are less than 0.7. Similar low ratios are common to urban stormwater and farmland drainage" (Geldreich, 1972).

The obvious major weakness of this approach is that, unless the FC and FS die off at identical rates, the FC/FS ratio will gradually change and will no longer reflect the original ratio in the fresh fecal material. Geldreich and Kenner (1969) made note of this fact and recommended that the FC/FS ratio was only valid during the first 24 hours immediately following the discharge of bacteria into the stream. However, it is not always possible to judge the age of the pollution and, even if one can, one cannot always estimate the time between excretion and discharge into the stream. As a result McFetters et al. (1974) concluded that the FC/FS ratio "is no longer of significance in determining the source of the contamination when considering bacteria that originate from domestic sewage".

McFetters et al. (1974) found that enterococci survive better than FC which survive better than S. bovis and S. equinus. Feachem (1975) has proposed that this differential die-away can in fact strengthen the value of the FC/FS ratio as a means of distinguishing human from non-human pollution. For example, in fecal material in which enterococci are the dominant FS group (as in human feces) the FC/FS ratio will tend to fall whereas in fecal material in which S. bovis and S. equinus dominate (for instance in cattle and pig feces as suggested by Deibel, 1964; McFetters et al. 1974; Raibaud et al. 1961). the FC/FS ratio will tend to rise. A predominantly human source should exhibit an initially high (>4) ratio which should then fall whereas a non-human source should exhibit an initially low ratio (<0.7) which should subsequently rise. Feachem's conclusions are summarized in the following table.

Table 2. Fecal source related to FC/FS ratios

Initial FC/FS ratio	Change through time of FC/FS ratio	Probable fecal source
> 4	Rise Fall	Uncertain Human
< 0.7	Rise Fall	Nonhuman Uncertain

Pseudomonas aeruginosa

This organism is an opportunistic pathogen of man and animals which may be spread by water. Although growth of the organism in water may occur under certain conditions, the major source of P. aeruginosa in waters appears to be fecal wastes of man and of animals associated with man. Studies have shown that there frequently appears to be little relation between populations of P. aeruginosa and those of other pathogens or fecal indicators (Hoadley, 1977).

Characterization of Pseudomonas aeruginosa isolated from waste-waters may also be of significant value in source determination because this species is found primarily in human as opposed to other domestic animal wastes; i.e. dogs and cats (Seyfried, Harris and Young, 1986 unpublished data; Wheater et al. 1979) and because serotyping allows for sub-speciation of this organism into approximately 17 different heat-stable somatic antigenic groups (Kusama, 1978). Source tracing of Pseudomonas aeruginosa infections by serotyping has been used successfully in many clinical trials (Young and Moody, 1974; Baltimore et al. 1974). The method has also been used to trace the source of infection in swimmers (Seyfried and Fraser, 1978) and could possibly be adapted to tracing studies in other environmental settings.

Bifidobacterium spp.

It has been suggested that Bifidobacterium spp. are good indicators of human fecal wastes in surface waters (Buchanan and Gibbon, 1947; Leven, 1977; and Resnick and Leven, 1981). Bifidobacteria are present in concentrations of 10^9 organisms per gram of feces in humans (Geldreich, 1979) but have a very limited distribution among other animals (Mara and Oragui, 1983). They have also been recovered from raw sewage (Resnick and Leven, 1981). Mara and Oragui (1983) reported that sorbitol fermenting species of bifidobacteria were exclusive to human fecal wastes and proposed a membrane filtration method for recovering bifidobacteria from surface waters.

Clostridium perfringens

Clostridium perfringens was suggested as an indicator of the pollution of water with fecal wastes in the late 1890s (Cabelli, 1977). However, because of the organism's extreme resistance, abundance in decomposing organic matter and soil, and failure to occur in numbers that correlate with the results of a sanitary survey (Levine, 1921) it is rarely used as an indicator. Nonetheless, the enumeration of C. perfringens spores as a water quality indicator appears to have specific and limited applications, primarily as an adjunct to the commonly used coliform and fecal streptococcus indicators.

C. perfringens is an indicator of choice when the requirement is for measuring remote and intermittent sources of pollution reaching an area (such as, tracing sewage sludge dumped into bodies of water or the infiltration of ground water supplies with fecal wastes) and in instances where other microbial indicators are rapidly destroyed, such as chlorinated water supplies.

Scope

In order to determine the relative merits of the novel source determination methods and to devise a methodology for the detection of human fecal wastes in storm sewer lines, a study to characterize the bacterial populations found in urban storm and sanitary wastes was initiated in the fall of 1986. Fecal indicator bacteria were enumerated in sanitary, storm and priority storm sewage and storm water runoff. Fecal streptococci and *Pseudomonas aeruginosa* were isolated and identified according to the phenotypes, serotypes and genotypes present in the different sample types. Bifidobacteria were also enumerated from storm and sanitary wastes and recovered isolates were tested for sorbitol fermentation.

METHODS

Sampling Sites

Sites A, B and C (shown in Table 3 and Fig. 1) in the Mount Steven Trunk storm sewer line were sampled because this area was designated a high priority sewer by the Ministry of the Environment. The non-priority sites, selected for comparison, were X, Y and Z in the Mount Steven Trunk storm sewer branch lines. During periods of wet weather, storm water run-off was also collected at X, Y and Z sites. These samples were labelled R, G. and Q, respectively. Samples D, E and F were obtained from a sanitary sewer in close proximity to the priority storm sewer sampling points.

Sample Collection

During periods of dry weather, triplicate samples were collected from each sampling point in the sewers over a two to four-day period. The dry weather samples were obtained in October, 1986; June, 1987; August, 1987; June, 1988; and August, 1988. Wet weather samples were collected from the sewers and the street run-off during rainy days in July, September and October, 1987. (See Appendix Table 14 for precise sampling dates).

Table 3. Sampling locations of high priority and non-priority storm sewers, sanitary sewer and storm water runoff.

Sample Description	Code	Site
High Priority Storm Sewer Line, Mount Steven Storm Sewer Trunk	A	Danforth and Jones Avenues - furthest in-line sampling point (near source of suspected solution input).
	B	Pape and Strathcona Avenues (mid-line sampling point).
	C	First and Broadview Avenues (near outfall).
Non-Priority Storm Sewer Branch Lines	Y	Chatham and Jones Avenues (connects to main line above sampling point B).
	X	Danforth and Woodycrest Avenues (connects to main line above sampling point A).
	Z	Pape and Cavell Avenues.
Storm Water Runoff	G	Chatham and Jones.
	R	Danforth and Woodycrest Avenues
	Q	Pape and Cavell Avenues
Sanitary Sewage Line	D	Danforth and Jones Avenues
	E	Strathcona and Pape Avenues
	F	First and Broadview Avenues

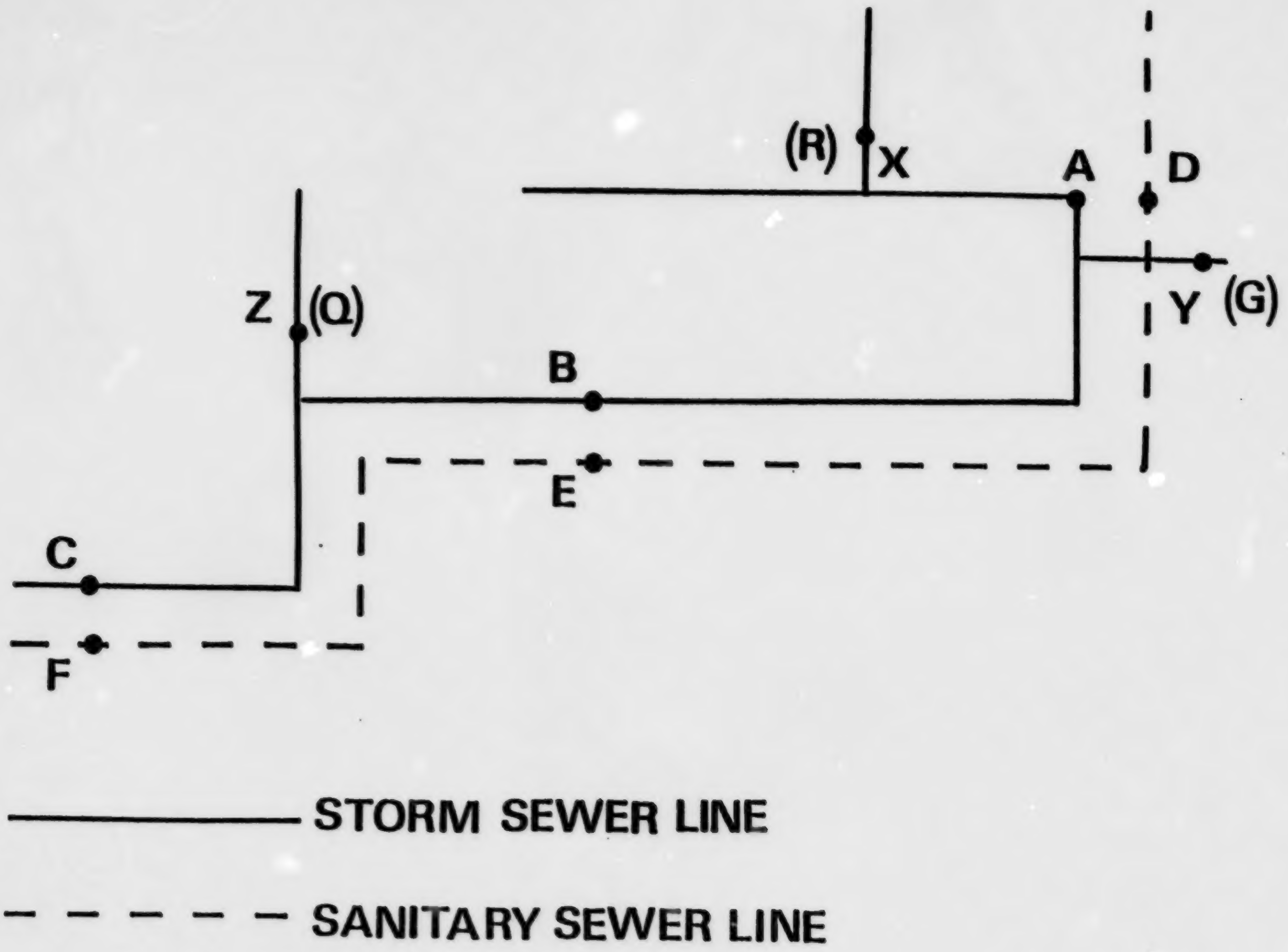
(R)
X
A
D
Y
(G)
Z
(Q)
B
E
C
F
STORM SEWER LINE
SANITARY SEWER LINE

FIG. 1 - Schematic diagram of Mount Steven storm sewer and sanitary sewer lines.

The samples were collected in sterile glass containers, transported to the laboratory on ice, and processed within 6 hours of collection.

Fecal specimens from both humans and animals were also obtained.

Bacterial Isolation and Enumeration

Analysis of the samples for indicator bacteria was by membrane filtration of appropriate dilutions of the samples through Gelman GN6 47 mm cellulose nitrate filters with a pore size of 0.45 um (Standard Methods, 1985). The filters were planted on media appropriate for the recovery of the various indicator bacteria.

Fecal coliform bacterial densities were determined by planting the filters on m-TEC agar (Dufour et al. 1981) and incubating for 23 ± 1 hours at 44.5 ± 0.5° C. Both target and non-target colonies were counted. Target colonies were yellow, yellow-green and yellow-brown; non-target colonies were blue to blue-green in colour. To ensure the accuracy of the counts, only results obtained from filters with target counts between 10 and 100 colonies were used to calculate the bacterial density per 100 mL of water sample.

A second step for the determination of E. coli by urease treatment (Dufour and Cabelli, 1975) was incorporated into the m-TEC procedure. Filters with appropriate target counts (i.e. between 10 and 100 target colonies) were removed from the m-TEC plates and placed on filter pads soaked in a urea phenol red solution (Dufour and Cabelli, 1975). The filters remained in contact with the filter pad for 15 min. to allow for deaminization by non-E. coli coliform bacteria processing urease. A second count of all urease-negative colonies (all yellow, yellow-green and yellow-brown colonies) was taken.

Fecal streptococci determinations were made by planting the filters on m-

Enterococcus agar (Difco) (APHA, 1985). The medium was incubated for 48 hr. at 35°C and a count of all pink to purple target colonies taken. Upper and lower counting limits of 10 to 150 target organisms were applied to the reported results.

Enterococci were recovered on m-ME agar (APHA, 1985) which also contains indoxyl-B-D-glucoside (IG). The m-ME plates were incubated for 48 hours at 41.5 ± 0.5° C. The incubation time was modified from the original 24 hr. suggested by Dufour as it allowed for a slight increase in recovery of target organisms. The addition of IG to the medium facilitates differentiation of the B-D-glucosidase enterococci from other fecal streptococci. Both target and non-target colonies were enumerated. Targeted colonies on m-ME were purple, white-blue to dark blue with blue haloes from degradation of the IG. Non-targets were pink to maroon non-haloed colonies. Counting range limits of 10 and 150 were also applied to these results.

Pseudomonas aeruginosa densities were determined using m-PA agar (APHA, 1985). The medium was incubated at 41.5 ± 0.5° C for 48 hr. and a count of all flat spreading brownish-green or tan colonies obtained. Upper and lower counting limits of 10 and 150 were applied to the results.

Bifidobacterium spp. were isolated on the YN-17 medium described by Mara and Oragui (1983). Target bifidobacteria colonies appear dark blue to black. Greenish coloured colonies were not counted; however, to ensure that all bifidobacteria were enumerated, crystal violet stains were made of colonies which had a different size or colour. Adjustments to the count were made if necessary.

Clostridium perfringens was isolated using a medium originally developed by Bisson and Cabelli (1979) and modified by the Ministry of the Environment

Southeastern Region Laboratory (m-CP2) (M.O.E., 1986). The samples were pretreated at 70° C for 15 or 30 min. to destroy the vegetative cells. Appropriate volumes of the samples were passed through membrane filters and the filters were placed on m-CP2 plates. The plates were then incubated anaerobically at 37° C for 48 hr. Target colonies appear yellow with a large black centre which can extend fairly close to the circumference; the colonies do not possess a blue halo. Nontarget colonies have a blue halo and they can be yellow or yellow with a black centre.

Formulations for the aforementioned media are provided in the Appendix.

All bacteria were identified to the species level using standard taxonomic methods.

Bacterial Characterization

Approximately 2,500 fecal streptococci were recovered on m-Enterococcus agar and m-ME agar during the dry and wet weather surveys. The isolates that were identified as varieties of *S. faecalis* were tested for their reaction in litmus milk broth (Difco) using the method of Mundt (1973).

Serotyping of the *P. aeruginosa* isolates was carried out using a Pseudomonas Antisera Kit (Difco). The organisms were sub-speciated into the 17 different heat-stable somatic antigen groups described by Kusama (1978).

Genotyping

For the restriction enzyme analysis (REA) of *P. aeruginosa*, total cellular DNA was extracted using a method described by Bradbury *et al.* (1984, 1985). A 1.5 mL volume of an 18 hour nutrient broth culture inoculated with *P. aeruginosa* was transferred into an Eppendorf tube and centrifuged in a

Microfuge 12 (Beckman) for 3 minutes at 7500 x g. The supernatant was discarded and the pellet loosened by vortexing. A 291 uL volume of PEB I buffer containing 10 mg/mL lysozyme was added and the mixture incubated for 20 minutes at 35° C. A 9 uL amount of 5M NaCl was added, thoroughly mixed, and then 150 uL of 10% sodium dodecyl sulfate (SDS) was added. The solution was gently mixed and incubated for 10 minutes at 37° C. Following the addition of 450 uL phenol:chloroform:isoamyl (25:24:1), the mixture was vortexed and centrifuged at 7500 x g for six minutes at room temperature. The upper aqueous phase was removed with a pasteur pipette and transferred to an Eppendorf tube. One mL of 95% cold ethanol was added, the tubes vigorously shaken and stored at -20° C overnight. The mixture was centrifuged for 3 minutes at 12, 000 x g, the supernatant discarded and the pellet redissolved in 250 mL DNA wash buffer. A total of 500 uL of 95% cold ethanol was added, the mixture stored at -20° C for 20 minutes, and centrifuged at 1,200 x g for 3 minutes. The supernatant was discarded and the pellet allowed to dry at 37° C for 10 minutes. The pellet was dissolved in 100 uL of distilled water and stored at 4° C until digested.

Restriction digests were performed using Sma I according to the manufacturer's instructions (Boerhinger Mannheim). A 10 uL aliquot of double strength (2X) Sma I buffer was placed in an Eppendorf tube and 10 uL extracted DNA added. A 2 uL sample of Sma I enzyme was added and the mixture was incubated for 1 hour at 37° C to allow for complete digestion. Following the addition of 1 uL of 0.15M EDTA + 0.4 mg/mL RNase A, the tube was incubated at 37° C for 20 minutes. A 5 uL volume of 5X sample buffer was then added to the restriction digest. Samples were electrophoresed on 0.7% agarose gel for 16 hours at 27 volts. Gels were stained with 1 mg/mL ethidium bromide in 1X TAE

(Tris base, 1.0 sodium acetate, 0.1 M disodium EDTA) for 1 hour and destained for 2 hours in distilled water. Photography was done using U.V. light at 300 nm and a red No. 23A polaroid 66p/N film with an exposure time of 30 seconds.

Fecal streptococci were grown in Brain Heart Infusion broth (Difco) at 37° C for 4 hours and the total cellular DNA extracted as described previously. Restriction digests were performed using Bam HI enzyme according to the manufacturer's directions (Boehringer Mannheim) along with a 10 uL aliquot of 2X Bam HI buffer.

Bifidobacterium sp. were grown anaerobically in MRS broth (Oxoid) for 48 hours at 37° C. The same method as was used for P. aeruginosa and fecal streptococci was employed to extract total cellular DNA from Bifidobacterium spp. When difficulties were experienced with the cellular DNA extraction procedure, the following treatments were added:

1. high concentration of lysozyme (i.e. 20 mg/mL);
2. sodium dodecyl sulfate (SDS) with normal lysozyme concentration;
3. alkaline lysis;
4. SDS with proteinase K;
5. proteinase K without SDS;
6. Triton X-100;
7. 4M guanidine isothiocynate; and
8. lysostaphase.

Three different enzymes, Sma I, Bam HI and Cfo, with their appropriate buffers, were used for the restriction enzyme analysis of the bifidobacteria.

RESULTS AND DISCUSSION

1986 and 1987 Surveys

The concentrations of fecal indicator bacteria and *Pseudomonas aeruginosa* found in sanitary sewage, high priority and non-priority storm sewage, and storm water runoff are presented in Tables 15 to 21 (Appendix). The results summarized in Table 4 show that the concentrations of fecal indicator bacteria in the Mount Steven Storm Trunk were high during both dry weather surveys. Although levels of greater than 10,000 fecal coliforms per 100 mL occurred throughout the main line (sampling points A, B, and C) high densities were noted most frequently at sites A and B. The source of the suspected fecal contamination is thought to be near site A, but the high fecal coliform and *E. coli* levels exhibited in sample B suggest that a second contaminant input may occur somewhere in-line between points A and B. The concentrations of fecal coliforms, and more specifically of *E. coli*, in sample C (near the outfall) tended to fluctuate but were usually lower than at sites A and B. In comparison, as shown in Figs. 2 and 3, the concentrations of fecal indicator bacteria in sanitary sewage were consistently high, as would be expected in this type of sample.

As may be seen in Table 22 (Appendix), the fecal coliform to fecal streptococcus ratios observed in the sanitary sewage samples in dry weather were generally above 4, indicating human fecal input. The FC/FS ratios varied from day-to-day in the storm sewer line, but there was a tendency toward ratios of > 4.0 at points A and B. At site C, the FC/FS ratios were always below 4 which would suggest that no source of contamination is present and that the area is being impacted upon by upstream pollution.

Table 4

Overall Geometric Mean Concentrations of Fecal Coliforms, *E. Coli*, Fecal Streptococci, Enterococci, *Pseudomonas aeruginosa*, Bifidobacteria and *Clostridium perfringens* Recovered from Sanitary Sewage as well as High Priority and Non-Priority Storm Sewage during Dry and Wet Weather Surveys.

Survey	Site	Fecal Coliforms	E. coli	Fecal Streptococci	Enterococci	Pseudomonas Aeruginosa	Bifidobacterium	Clostridium Perfringens
Dry Weather 1^a	Sanitary Sewage							
	D	2.0×10^6	7.1×10^3	2.0×10^3	2.0×10^3	1.2×10^1*	-	-
	E	2.7×10^6	4.0×10^3	1.6×10^3	2.0×10^3	2.2×10^1*	-	-
	F	3.9×10^6	2.2×10^3	2.2×10^3	8.5×10^2	4.6	-	-
	High Priority Storm Sewage							
	A	1.4×10^6	8.1×10^3	1.5×10^5	1.4×10^5	8.6×10^3	-	-
	B	1.5×10^6	1.3×10^4	1.9×10^5	9.4×10^4	4.5×10^3	-	-
	C	2.3×10^6	3.8×10^3	3.8×10^5	4.9×10^5	2.7×10^3*	-	-
Dry Weather II^a	Sanitary Sewage							
	D	2.0×10^6	1.9×10^6	1.5×10^4	5.0×10^4	1.0×10^4	3.7×10^4	2.3×10^4
	E	3.5×10^6	2.6×10^6	1.1×10^4	1.1×10^5	1.8×10^4	6.3×10^4	5.2×10^4
	F	1.7×10^6	1.5×10^6	2.75×10^5*	2.4×10^5	8.4×10^3	7.1×10^4	1.0×10^4
	High Priority Storm Sewage							
	A	4.9×10^4	3.1×10^4	1.0×10^4	3.9×10^3	1.6×10^2	5.2×10^4	6.4×10^2
	B	8.5×10^4	6.2×10^4	8.0×10^3	2.4×10^3	6.3×10^2	1.4×10^5	4.4×10^2
	C	9.3×10^3	6.0×10^3	4.8×10^4	1.3×10^4	9.2×10^1	1.5×10^3	7.5×10^2
	Y	3.8×10^3	2.9×10^3	4.0×10^3	2.5×10^3	3.9×10^1*	5.2×10^2	5.35×10^2
	Non-Priority Storm Sewage							
	X	1.3×10^3	9.0×10^2	3.5×10^3	2.1×10^3	6.75	1.2×10^1*	1.2×10^2
Wet Weather 1^b	Runoff							
	P	5.8×10^3	4.8×10^3	2.4×10^4	-	5.6×10^2	-	-
	Q	6.3×10^3	2.9×10^3	3.0×10^3	-	4.6×10^1	-	-
	Non-Priority Storm Sewage							
	X	1.3×10^5	8.6×10^4	8.9×10^4	9.3×10^4	1.5×10^3	2.4×10^3	1.3×10^3
	Z	4.1×10^4	1.1×10^4	1.2×10^5	7.1×10^4	5.1×10^2	4.4×10^2	-

* Approximate Data. [a] Sampled over a 4-day period; [b] Sampled over a 1-day period.

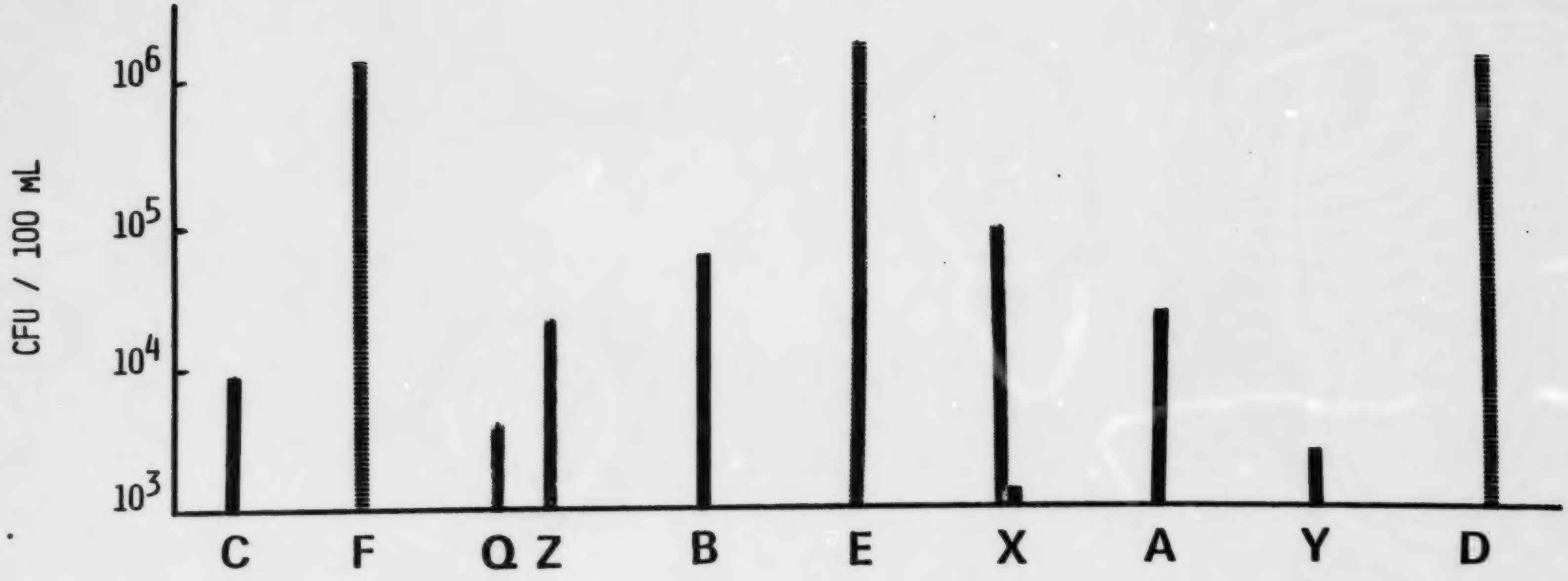

Fig. 2 Fecal coliform levels at the storm sewer and sanitary sewer sampling sites.

Refer to Table 3 for site descriptions.
Solid line at site X represents counts during dry weather and dotted line represents wet weather counts.

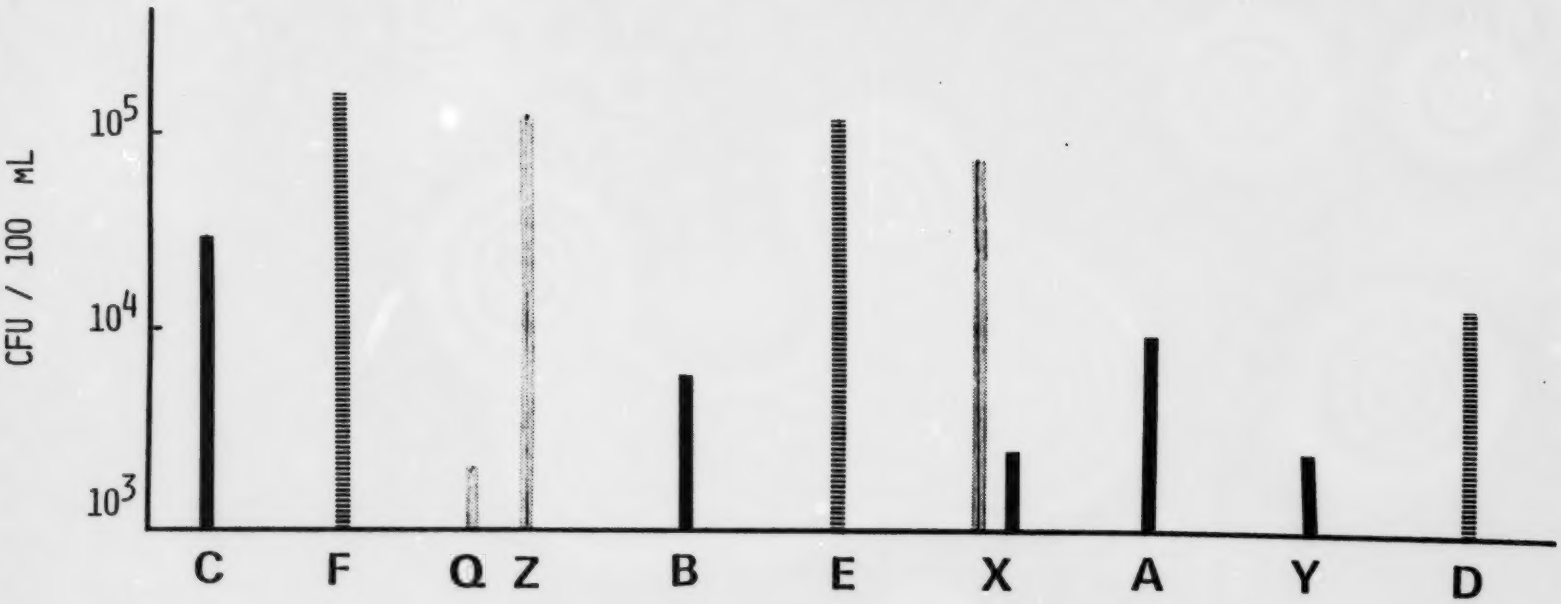

Fig. 3 Fecal streptococcus levels at the storm sewer and sanitary sewer sampling sites.

Pseudomonas aeruginosa was also present in high concentrations in the sanitary sewer (Fig. 4). Although this organism was found to occur in only 12% of the human population (Sutter et al., 1967), it is consistently isolated from sewage and highly polluted surface waters (Bonde, 1963; Hoadley, 1967; Cabelli, Kennedy and Levin, 1976).

Pseudomonas aeruginosa was recovered from the high priority storm sewer (A, B and C) during all dry weather surveys in 1986 and 1987 (Tables 15 to 20 Appendix). The levels exhibited during the second dry weather survey were higher than those recovered in survey 1 (Table 4) possibly due to the fact that the second survey was conducted during the summer period as opposed to the late fall sampling of survey 1. Pseudomonas has been shown to exhibit regrowth in nutrient enriched waters at high temperatures (Hoadley, 1977).

Previous investigators have shown P. aeruginosa to be more indicative of human rather than animal fecal wastes (Wheater et al., 1978, 1979). The concentrations present at Points A and B, particularly during dry weather survey 2, would suggest that the contaminate input at these sites is human in origin. Levels of Pseudomonas at point C tended to be lower than at points A and B, again confirming that the source of the contamination lies upstream of this point.

Pseudomonas aeruginosa was not isolated from the high priority storm sewer during the first dry weather survey on October 28. Rainfall on the previous evening may have caused dilution effect in the sewer such that P. aeruginosa could not be detected. Any incoming fecal material from street runoff would be of non-human origin (e.g. dog) (Geldreich, 1979). This would cause an increase in the fecal coliform, E. coli and fecal streptococcus concentrations in the sewer but would not necessarily increase P. aeruginosa concentrations since

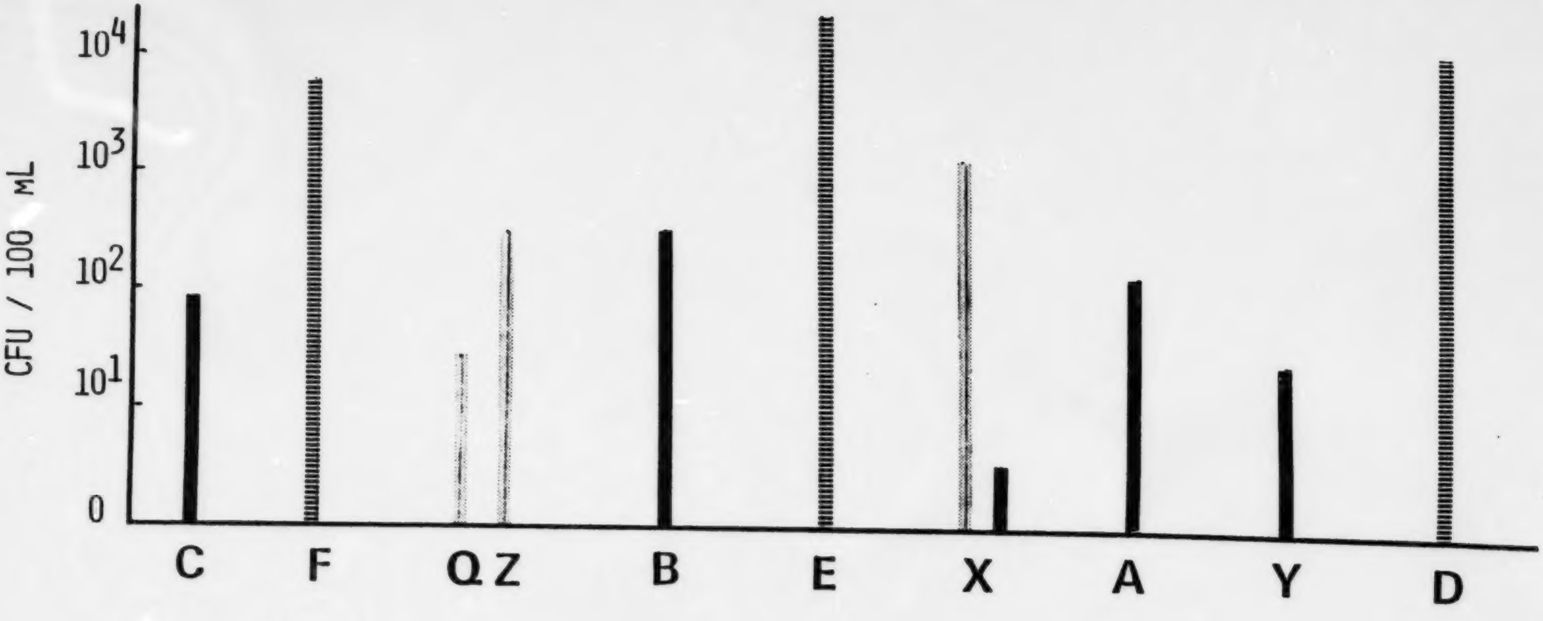

Fig. 4 Pseudomonas aeruginosa levels at the storm sewer and sanitary sewer sampling sites

animal feces (i.e. dogs, cats, raccoons) generally do not contain Pseudomonas (Seyfried, Harris and Young, manuscript in preparation).

Bifidobacteria was recovered from both high priority storm and sanitary sewage during the dry weather surveys (Tables 15 to 20, Appendix). This organism is thought to occur primarily in human wastes (Mara and Oragui, 1983) and was found in concentrations of 10^8 to 10^{10} in the human feces analyzed as a separate facet in this study. The high concentrations of this bacterium in the storm sewer at points A and B and the lower concentrations found at point C (Fig. 5) again confirms that human fecal input occurs at the two upstream points because this organism exhibits rapid die-off in surface waters (Oragui, 1982).

Clostridium perfringens was included as a parameter during the second dry weather survey. Current literature available on the bacterium suggests that it is associated with both human and animal wastes (Bisson and Cabelli, 1980; Geldreich, 1979). Generally the organism is regarded as a good indicator of fecal pollution in situations where there has been environmental stress due to disinfection, prolonged transit time or the presence of toxic wastes (Bisson and Cabelli, 1980; Geldreich, 1979; Fujioka and Shizumura, 1985). C. perfringens was recovered from both high priority storm and sanitary sewage, and was present in higher concentrations in sanitary sewage (Fig. 6) although it could not be isolated from the human fecal samples assayed. According to Geldreich (1979), C. perfringens is found in concentrations of 10^6 to 10^7 per gram of human feces but is only present in 13 to 35 percent of the population.

Additional samples X and Y analyzed during the second dry weather survey were collected from branch lines connecting to the main trunk line (see Table 3 and Fig. 1). These lines were thought to be non-priority storm sewage

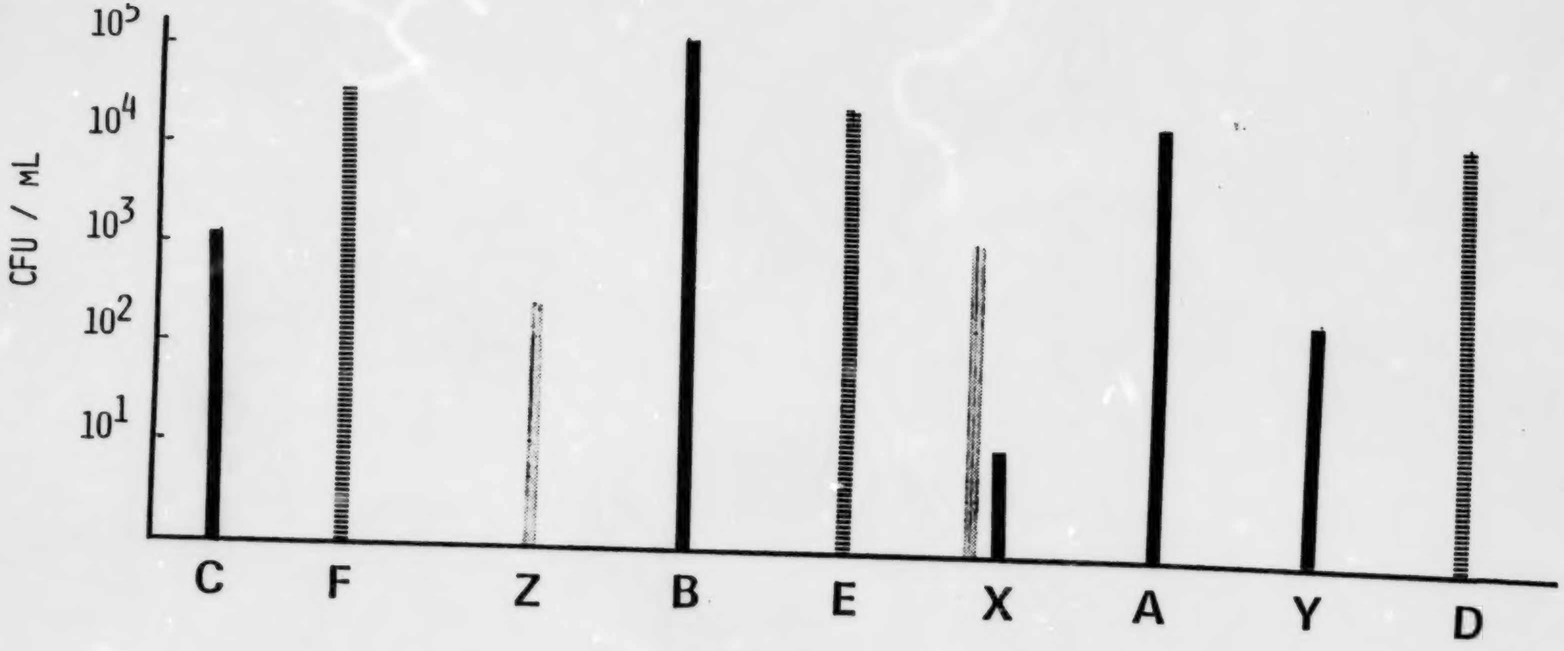

Fig. 5 Bifidobacteria levels at the storm sewer and sanitary sewer sampling sites

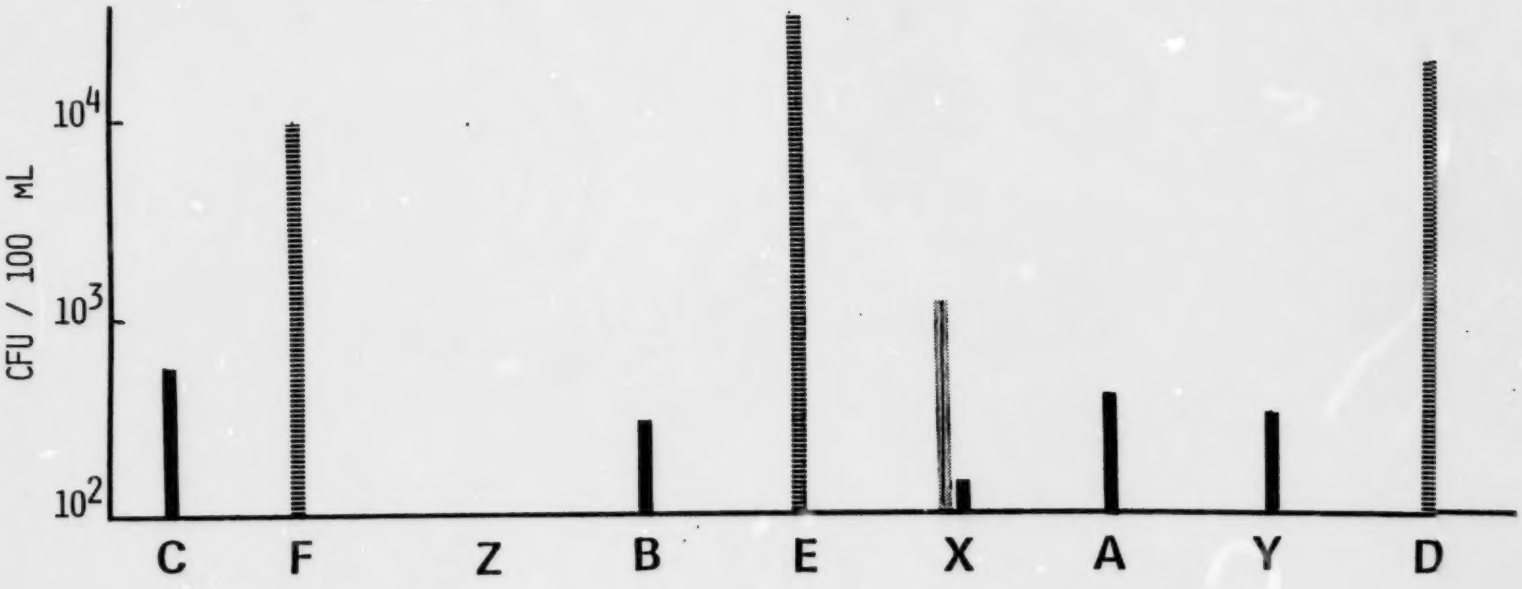

Fig. 6 *Clostridium perfringens* levels at the storm sewer and sanitary sewer sampling sites

according to the City of Toronto Public Works Department. Sample X exhibited low fecal indicator counts as would be expected in non-priority storm sewage. The sample also contained very low Pseudomonas and Bifidobacterium levels which would indicate the absence of human fecal input at this site. Sample Y, however, exhibited high concentrations of fecal coliforms and E. coli, and on June 12 the FC counts were greater than 10,000/100 mL. Concentrations of Pseudomonas aeruginosa and Bifidobacterium were also high in this sample suggesting that a contaminant input, possibly of human origin is impacting on the sewer at or near this site. Although site Y is located upstream of site B, (see Fig. 1) it cannot account for the magnitude of pollution exhibited at B since the bacterial concentrations recovered at Y were lower than those presentat the B site. Because of its high fecal indicator bacterial levels, sample Y was grouped with the high priority storm sewage.

The FC/Fs ratios exhibited in samples X and Y were generally below 4. This would appear to contradict the evidence of human fecal input at site Y, i.e. Bifidobacteria concentrations. Previous studies however, have shown that FC/FS ratios tend to fluctuate over time as a result of the different die-off rates of the two bacterial groups (McNeil, 1985). It may be that the fecal pollution input impacting on the sewer at site Y is located in-line above this sampling site. Since Bifidobacteria also tend to die-off rapidly (Oragui, 1982), it would be interesting to determine if samples taken above site Y in this branch line exhibited an increase in this organism as well as an increase in the FC/FS ration and levels of other fecal indicator bacteria.

Fecal indicator bacterial concentrations recovered from non-priority storm sewage and storm water runoff during wet weather (Table 4) revealed that street runoff is heavily contaminated with fecal material and contributed greatly to the contamination in storm sewer lines during storm events. The rainfall for this event commenced on the afternoon of July 13 and continued until the late morning of July 14. Samples Q, X and Z were collected after the first flush had occurred. Sample P was collected from street water runoff at Parliament and Carlton streets at the start of the rainfall event and this may account for the higher *Pseudomonas* levels exhibited in this storm water runoff sample over that of storm water sample Q. As well, the fact that sample P was collected from a different geographical location may account for this difference.

Allen Gardens is an area where gulls and pigeons feed and both of these bird species were found to carry *Pseudomonas* (Seyfried, Harris and Young, 1986, unpublished data). During wet weather, *Pseudomonas aeruginosa* concentrations in storm water and storm sewage may also be increased by runoff from vegetation (Hoadley, 1977).

The fecal coliform to fecal streptococci ratios exhibited in storm water and storm sewage during wet survey 1, were all below 4.0 suggesting fecal input of non-human origin. It would appear that FC/FS ratios may still be a useful diagnostic tool for source determination in storm sewer lines when applied close to the input, although many investigators cautioned its use in surface water analyses (Wheater *et al*., 1979; Palmer 1984; Diebel 1964).

Bifidobacteria were recovered from non-priority storm sewage samples X and Z during wet weather. Concentrations exhibited in sample X were much higher than those recovered during dry weather at this site. Our studies showed that animals such as dogs can carry Bifidobacteria in their feces.

The distribution of fecal streptococcal populations recovered from storm and sanitary sewage during the dry and wet weather surveys is given in Tables 23 to 31 (Appendix). The results from dry weather survey 2 are summarized in

Table 5. Streptococcus faecium comprised the greatest percentage of the fecal streptococcal group found in storm sewage and sanitary sewage during dry weather with the exception mainly of samples B and F on a few sampling days. Seyfried, Harris and Young (1986 unpublished data) found S. faecium to be predominant in human fecal samples as well as several animal species (i.e. dogs, raccoons). Studies by Wheater et al. (1979) have also shown S. faecium to predominate in human feces and sanitary sewage. However, other workers (Cooper and Ramadan, 1955; Kenner, 1978; Kjellander, 1960; and Mundt, 1982) have found a greater percentage of S. faecalis varieties. Dietary differences have been shown to account for variations in the streptococcal flora of the human intestinal tract (Hill et al., 1971; Finegold et al., 1975). There is a tendency for S. faecium to survive for longer periods than S. faecalis in polluted waters (Dufour 1985 personal communication) which may account for its greater recovery from storm and sanitary sewage. Within the S. faecalis group there was some variation in recovery of S. faecalis var. faecalis and S. faecalis var. liquefaciens in the storm and sanitary sewage samples, with both varieties predominating during different dry weather surveys and at different sample points in both sewers. Other workers have found S. faecalis var. faecalis to be dominant in human feces (Kenner, 1978). Geldreich (1979) found that S. faecalis var. liquefaciens was present in only 26 percent of the human population and could be recovered from environmental sources. Site X (non-priority) exhibited low percentage of S. faecalis varieties and showed a higher percentage of S. faecium var. casseliflavus an organism which is found on vegetation (Mundt and Graham, 1968) and in the feces of animals such as geese (Seyfried, Harris and Young, 1986, unpublished data). S. faecium var. casseliflavus was also recovered in high percentages in storm water and storm

Table 5

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather Survey 2, 1987

Species and variant	High Priority Storm Sewage			Non-Priority Storm Sewage		Sanitary Sewage		
	A [125]	B [120]	C [124]	Y [116]	X [112]	D [141]	E [144]	F [106]
S. faecalis var.								
faecalis	11 (8.8)	41 (34.2)	15 (12.1)	16 (13.8)	5 (4.5)	19 (13.8)	12 (8.3)	17 (16.0)
liquefaciens	6 (4.8)	12 (10.0)	18 (14.5)	22 (19.0)	4 (3.6)	20 (14.2)	32 (22.2)	28 (26.4)
zymogenes	3 (2.4)	-	1 (0.8)	7 (6.0)	1 (0.9)	-	9 (6.2)	2 (1.9)
S. faecium	36 (28.8)	32 (26.7)	32 (25.8)	36 (31.0)	32 (28.6)	86 (61.0)	85 (59.0)	57 (54.0)
S. faecium var. casseliflavus	31 (24.8)	12 (10.0)	16 (12.9)	23 (19.8)	56 (50.0)	4 (2.8)	3 (2.1)	1 (0.9)
S. durans	3 (2.4)	4 (3.3)	8 (6.4)	4 (3.4)	4 (3.6)	9 (6.4)	1 (0.7)	-
S. bovis (A type)	8 (6.4)	3 (2.4)	1 (0.8)	2 (1.7)	-	-	-	-
S. avium	14 (11.2)	11 (9.2)	9 (7.2)	2 (1.7)	1 (0.9)	2 (1.4)	-	-
FS	5 (4.0)	1 (0.8)	10 (8.1)	-	6 (5.3)	-	2 (1.4)	
non FS	7 (5.6)	2 (1.7)	7 (5.6)	1 (0.9)	3 (2.7)	1 (0.7)	-	-
S. lactis	1 (0.8)	2 (1.7)	6 (4.8)	2 (1.7)	-	-	-	-
S. equinus				1 (0.9)				
S. galactis			1 (0.8)					1 (0.9)

( ) percentage of total
[ ] number of isolates identified

sewage during wet weather but was generally not recovered from sanitary sewage and has not been shown to occur in human feces (Seyfried, Harris, and Young, 1986 unpublished data). Other streptococci species not recovered in sanitary sewage but found in storm sewage were S. bovis and S. avium. Both of these species are found in animals such as dogs but not in human fecal material (Seyfried, Harris and Young, 1986 unpublished data).

The litmus milk reactions of S. faecalis varieties summarized in Tables 6 and 7 (from Tables 32 to 40, Appendix) demonstrate that acid curd producing strains are common in sanitary sewage. Mundt (1973) found that over 90% of S. faecalis cultures from non-human sources such as animals, plants and insects gave proteinization reactions in litmus milk, while isolates from human feces produced an acid curd. Seyfried et al. (unpublished data) also found that isolates of S. faecalis from humans did not proteinize litmus milk, However, these same isolates also did not demonstrate the ability to produce acid curd reactions.

Most of the isolates from sanitary sewage giving proteinization reactions were S. faecalis var. liquefaciens and were probably of non-human origin because this variety can occur on such environmental sources as plants (Mundt et al., 1959). Isolates from high priority storm sewage showed a high percentage of acid curd production, but this reaction was also given by some of the non-priority storm sewage isolates from sample X during dry and wet weather and by isolates obtained from storm water sample Q. Although storm water and non-priority storm sewage S. faecalis isolates will have to be tested before the usefulness of this test can be assessed, it would appear that the litmus milk reactions are too nonspecific to be of value.

Table 6

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather Survey 1

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	No Reaction
High priority Storm Sewage						
A	35	13 (37.1)	2 (5.7)	-	4 (11.4)	16 (45.7)
B	31	6 (19.3)	5 (16.1)	-	12 (38.7)	8 (25.8)
C	19	9 (47.4)	1 (5.3)	1 (5.3)	6 (31.6)	2 (10.5)
Sanitary sewage						
D	28	24 (85.7)	-	1 (3.6)	-	3 (10.7)
E	30	23 (76.7)	1 (3.3)	2 (6.7)	-	4 (13.3)
F	63	50 (79.4)	2 (3.2)	5 (7.9)	2 (3.2)	4 (6.3)

( ) percentage

Table 7

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather 2 and Wet Weather Survey 1 Samples

Survey	Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	No Reaction
Dry Weather Survey 2	High priority Storm Sewage						
	A	20	12 (60.0)	-	1 (5.0)	6 (30.0)	1 (5.0)
	B	53	44 (83.0)	3 (5.7)	-	4 (7.5)	2 (3.8)
	C	34	19 (55.9)	1 (2.9)	2 (5.9)	10 (29.4)	2 (5.9)
	Y	45	27 (60.0)	-	1 (2.2)	14 (31.1)	3 (6.7)
	Non-priority Storm Sewage						
	X	10	3 (30.0)	-	1 (10.0)	4 (40.0)	2 (20.0)
	Sanitary sewage						
	D	39	24 (61.5)	-	1 (2.6)	1 (2.6)	13 (33.3)
	E	53	15 (28.3)	-	2 (3.8)	29 54.7)	7 (13.2)
	F	47	26 (55.3)	-	-	19 (40.4)	2 (4.2)
Wet Weather Survey 1	Storm Water Runoff P	10	2 (20.0)	2 (20.0)	2 (20.0)	3 (30.0)	1 (10.0)
	Q	21	11 (52.4)	2 (9.5)	3 (14.3)	1 (4.8)	4 (19.0)
	Storm Sewage						
	X	18	8 (44.4)	-	1 (5.6)	8 (44.4)	1 (5.6)
	Z	12	1 (8.3)	1 (8.3)	5 (41.7)	4 (33.3)	1 (8.3)

( ) percentage

The greatest percentage of Pseudomonas aeruginosa serotypes in high priority and non-priority storm sewage, sanitary sewage and storm water runoff were serotype number 6 (Tables 41 to 50, Appendix). Other serotypes common to all sample types were serotypes 1, 10, 11, 4, 3, and 2. Previous research has shown most of these isolates to be common to both humans and animals (Habs 1957; Sandvik 1960; and Verder and Evans 1961). On occasion, serotype 10 was found to be more prevalent in storm sewer sample Y than was serotype 6. This serotype was rarely isolated from non-priority storm sewage and storm water runoff and was isolated from sanitary sewage but not to the degree that would be expected if this serotype was common to fecal material. Overall, the value of Pseudomonas serotyping as a means of pollution source differentiation appears to be limited and perhaps it would be more useful to look at Pseudomonas concentrations in storm sewage.

The percentage of sorbitol fermenting Bifidobacteria in human feces and sanitary sewage is high (Table 8). Mara and Oragui (1983) have found sorbitol fermenting species of Bifidobacteria, i.e. B. adolescentis and B. breve to be exclusive to human feces. Other workers (McNeil 1985) have also reported this fact. The presence of these organisms in human feces is not affected by diet and geographical variation (Drasor 1974) as are group D streptococci.

Bifidobacteria isolates obtained from dog and cat feces did not ferment sorbitol. Although some of the non-priority storm sewage isolates gave sorbitol fermentation reactions not enough isolates were tested (i.e. sample X) to make any definite conclusions about the results. Isolates from high priority storm sewage samples A and B showed somewhat higher percentages of sorbitol fermentation reactions. Sample Y, which was originally submitted as non-priority storm sewage showed a higher percentage of the sorbitol

Table 8

Percentage of Sorbitol Fermenting Bifidobacteria in High Priority and Non-Priority Storm Sewage, Sanitary Sewage and Feces

Sample	Source	Total Isolates Identified	Number of Sorbitol Fermentors
D,E,F	Sanitary Sewage	37	24(65)
A	High Priority Storm Sewage	35	8(23)
B		29	4(14)
C		12	0
Y		32	13(41)
X	Non-Priority Storm Sewage	15	3(20)
Z		5	2(40)
Feces	Human	38	23(61)
	Dog	24	0
	Cat	18	0

( ) percentage

fermenting strains than high priroity storm sewage samples A and B and as previously mentioned, this site could possibly be impacted on by a human sanitary input.

It would appear, based on these results, that sorbitol fermenting Bifidobacteria may be good indicators of human fecal contamination.

SUMMARY

Summary of 1986-1987 Survey Results

1. Fecal contamination, most likely of human origin, is present in the high priority storm sewer line at or near sampling points A, B and Y.
2. Upstream contamination is impacting on the storm sewer at the downstream area C.
3. Street runoff during wet weather is highly contaminated with fecal material.
4. Bifidobacteria may be useful as indicators of human fecal wastes in storm sewage.
5. A high percentage of acid curd producing strains of S. faecalis may be indicative of sanitary wastes in storm sewer lines but the results are inconclusive.
6. FC/FS ratios may be useful as a supplementary interpretive tool for source differentiation within storm sewer lines. This would have to be investigated further because the results were not consistent at all points.
7. High Pseudomonas aeruginosa concentrations in storm sewage may indicate the presence of human sanitary wastes but Pseudomonas serotyping is not applicable to source differentiation.
8. Source determination during storm events cannot be accomplished with a

high degree of accuracy.

1988 Surveys

Data from the 1986-1987 surveys (Seyfried et al., 1987) suggested that fecal contamination, possibly human in origin, was evident in the storm sewer line near sites A and Y. The results of the 1988 surveys, presented in Tables 51 to 53 (Appendix) and summarized in Table 9, add support to this conclusion. As may be seen in the table, the fecal coliform levels in the high-priority storm sewer were greater than 10,000/100 mL at site A in June and at all sites in August. It should be noted that counts of all indicator organisms tended to be higher in August, possibly due to regrowth of the bacteria in the warmer nutrient-enriched waters (Hoadley, 1977).

As might be expected, fecal coliform and fecal streptococcus counts were highest in sanitary sewage and the fecal coliform to fecal streptococcus ratio was greater than 4 in these samples (see Table 22, Appendix). Although a ratio greater than 4 was observed at site A in the priority storm sewer, at other storm sewer sites the FC/FS ratios were generally below 4 suggesting that there was little or no human fecal input (Geldreich and Kenner, 1969).

P. aeruginosa and Bifidobacterium sp. were shown in the 1987 study to have potential as indicators of human fecal waste. Collaborative data presented in Table 9 shows that counts of both organisms were higher in sanitary and priority storm sewage than in non-priority storm sewage.

Mara and Oragui first proposed the use of sorbitol fermenting bifidobacteria as indicators of human fecal pollution in 1983. Kator and Rhodes (1988) also used these organisms to differentiate human from animal sources of pollution in shellfish growing waters. The species of Bifidobacterium that are reportedly human specific and sorbitol fermenting are

Table 9

Overall Geometric Mean Concentrations of Fecal Coliforms, E. Coli, Fecal Streptococci, Enterococci, Pseudomonas aeruginosa, and Bifidobacteria Recovered from Sanitary Sewage as well as High Priority and Non-Priority Storm Sewage during two Dry Weather Surveys in 1988.

Survey	Site	Fecal Coliforms	E. coli	Fecal Streptococci	Enterococci	Pseudomonas aeruginosa	Bifidobacterium
Dry Weather June, 1988	Sanitary Sewage						
	D	9.77×10^{5}	6.91×10^{5}	1.74×10^{5}	1.45×10^{5}	2.69×10^{4}	NA
	E	1.00×10^{6}	8.51×10^{5}	1.90×10^{3}	2.75×10^{3}	7.08×10^{3}	NA
	F	3.31×10^{4}	3.31×10^{4}	1.51×10^{4}	6.46×10^{3}	1.00×10^{4}	1.58×10^{4}
	High Priority Storm Sewage						
	A	1.57×10^{4}	5.94×10^{3}	6.80×10^{3}	1.31×10^{3}	2.62×10^{2}	NA
	B	5.88×10^{3}	3.72×10^{3}	9.35×10^{3}	4.33×10^{3}	1.90×10^{2}	NA
	C	5.62×10^{3}	3.09×10^{3}	7.43×10^{4}	5.77×10^{4}	2.75×10^{2}	NA
	Y	7.76×10^{2}	5.57×10^{2}	2.54×10^{3}	1.01×10^{3}	3.11×10^{1}	NA
	Non-Priority Storm Sewage						
	X	9.77×10^{2}	7.94×10^{2}	4.57×10^{4}	3.16×10^{4}	1.78×10^{1}	NA
	Z	2.29×10^{2}	1.48×10^{2}	8.13×10^{3}	2.75×10^{2}	1.29×10^{1}	7.59×10^{3}
Dry Weather August, 1988	Sanitary Sewage						
	D	1.53×10^{8}	1.35×10^{8}	2.64×10^{6}	1.26×10^{6}	2.68×10^{4}	NA
	E	1.05×10^{7}	6.30×10^{6}	2.26×10^{4}	6.85×10^{3}	2.82×10^{4}	NA
	F	1.42×10^{4}	9.56×10^{3}	2.43×10^{3}	1.76×10^{3}	5.65×10^{1}	5.8×10^{4}
	High Priority Storm Sewage						
	A	1.21×10^{5}	1.65×10^{4}	4.46×10^{4}	4.07×10^{4}	1.14×10^{3}	NA
	B	1.00×10^{4}	5.25×10^{3}	2.00×10^{3}	2.57×10^{2}	NA	NA
	C	2.64×10^{4}	1.48×10^{4}	7.59×10^{4}	3.83×10^{4}	1.18×10^{1}	1.26×10^{5}
	Y	5.41×10^{4}	2.13×10^{4}	5.27×10^{4}	5.25×10^{4}	1.26×10^{4}	2.29×10^{4}
	Non-Priority Storm Sewage						
	X	4.07×10^{5}	3.63×10^{4}	3.03×10^{3}	3.92×10^{2}	2.88×10^{3}	NA
	Z	2.88×10^{4}	1.02×10^{4}	3.24×10^{2}	2.34×10^{2}	3.80×10^{2}	NA

B. adolescentis and B. breve. In this study we were able to isolate B. breve and B. bifidum from human fecal material; however, we also recovered B. adolescentis from dog fecal samples and B. breve, B. minimum and B. thermophilum from chicken feces. Twenty-one isolates that were thought to be Bifidobacterium on the basis of their morphology were recovered from eight different sewage samples. Of the 21 isolates, only two could be identified by biochemical testing. The two were found in the non-priority storm sewer at site Z and were classified as mannose + and mannose - strains of B. thermophilum.

Based upon our prior use of restriction enzyme analysis to distinguish between different strains of Klebsiella pneumoniae (Seyfried et al., 1989), it was felt that genotyping might assist in determining the source of the Bifidobacterium strains under investigation. Three enzymes were used to digest whole cell DNA from B. adolescentis and two Bifidobacterium sp. isolated from chicken feces. Restriction (REA) patterns using the total cellular DNA restriction enzyme analysis were obtained. However, when we attempted to repeat the restriction enzyme analysis, using the three different enzymes and eight modifications of the procedure (as described in the Methods section), we were unable to obtain satisfactory REA patterns. It is probable that isolates from the YN-17 medium, thought to be Bifidobacterium sp., were actually streptococci because of the initial confusion concerning the colony description (see Discussion in the report by In-ja Huh in the Appendix).

Similar to the 1986 and 1987 survey results, serotyping of the P. aeruginosa 1988 survey isolates showed that serotype 6 predominated in the priority and non-priority storm sewage, sanitary sewage and storm water runoff (Tables 54 to 58, Appendix). Serotypes 1, 10, 11, 4, 3 and 2, although not as prevalent as 6, were also common in all categories of samples.

Seventy-eight strains of P. aeruginosa from the sanitary sewer and 112 strains recovered from the storm sewer and storm water runoff were also genotyped. Forty-six different REA patterns were noted among the 191 isolates. As may be seen from Table 10, there was an interesting distribution of patterns among the sample groups. For example, REA patterns 1', 6', 6' and 13' were found among isolates from the three sampling sites in the sanitary sewer. The fact that these same patterns or genotypes were also prevalent in the priority storm sewer samples suggests that they may be typical of human fecal isolates. A comparison of the corresponding serotypes for each genotype showed that the serotypes tended to be widely distributed. For example, the 1' genotype was found in serotypes 1, 6 and 10; REA pattern 6 was distributed among serotypes 1, 3, 4, 6 and 11; 6' occurred in serotypes 1, 6, 9, 10 and 11; and the 13' genotype was found in serotypes 6 and 10. Because it seemed unusual to find such a high number of genotype 6 isolates in the non-priority storm and runoff samples, the source of these organisms was examined. It was found that the bacteria were all isolated during the wet weather sampling in July, 1987 from sites X and P. The P site was an additional street runoff sample taken at the beginning of the rainfall event. These isolates, belonging to REA pattern 6, were evenly divided between seroytpes 1 and 6.

Additional information provided in Table 11 shows that genotypes 1, 2, 4, 7, 9, 11", 12 and 14 were all isolated from sanitary and priority storm sewers and not from non-priority storm sewers or storm water runoff.

In comparison P. aeruginosa isolates with genotypes 18 and 20 were found solely in the non-priority sewer and storm runoff samples. The REA pattern 18 organisms were distributed between serotypes 3 and 6, whereas pattern 20 was found in serotypes 1 and 3. Serotype 3, REA pattern 18 P. aeruginosa isolates

TABLE 10. Distribution of the prominent *Pseudomonas aeruginosa* REA patterns among sanitary sewer, priority and nonpriority storm sewer and storm water runoff samples.

REA Pattern 46[a]	Sanitary Sewer Sites D,E,F 78	Priority Storm Sewer Sites A,B,C,Y 49	Nonpriority Storm Sewer Sites X,Z 29	Storm Water Runoff Sites P,Q,G,R 35
1′	8 (10.2)*	7 (14.3)	0 (0.0)	2 (5.7)
6	6 (7.7)*	4 (8.2)*	5 (17.2)	10 (28.6)
6′	4 (5.1)	3 (6.1)	0 (0.0)	1 (2.80)
13′	8 (10.2)*	5 (10.2)	0 (0.0)	0 (0.0)
18	0 (0.0)	0 (0.0)	4 (13.8)*	6 (17.1)
20	0 (0.0)	0 (0.0)	2 (6.9)	3 (8.6)

[a] Total number of REA patterns or samples in each category.

* Found at all sites in the sample category (e.g. sanitary sewer, sites D, E and F).

() Percentage

Table 11

Distribution of *Pseudomonas aeruginosa* REA Patterns Among Sanitary Sewer, Priority and Non-Priority Storm Sewer and Storm Water Runoff Samples Collected during Periods of Wet and Dry Weather

REA Pattern	Sanitary Sewer Sites D, E, F	Priority Storm Sewer Sites A,B,C,	Y	Non-Priority Storm Storm Sewer Sites Wet X, Z	Storm Water Runoff Sites P,Q,G,R
1	4 (5.13)	1 (2.63)			
1'	8 (10.25)	4 (10.52)	3 (27.27)		2 (5.71)
1''			1 (9.09)		
2	1 (1.3)	2 (5.26)			
2'	1 (1.3)				
2''	1 (1.3)				1 (2.85)
3	3 (3.84)				
4	1 (1.3)	2 (5.26)			
5	10 (12.82)	2 (5.26)	1 (9.09)	1 (3.44)	1 (2.85)
5'	1 (1.3)				
6	6 (7.69)	3 (7.89)	1 (9.09)	5 (17.24)	10 (28.57)
6'	4 (5.13)	3 (7.89)			1 (2.85)
6''					1 (2.85)
7	5 (6.41)	1 (2.63)			
7'	3 (3.84)			1 (3.44)	2 (5.71)
7''	2 (2.56)				
8	4 (5.13)				
8'	1 (1.3)		1 (9.09)	1 (3.44)	
8''	2 (2.56)				
8'''		1 (2.63)			1 (2.85)
9	1 (1.3)	2 (5.26)			
10	1 (1.3)				
10'	2 (2.56)	1 (2.63)	1 (9.09)	3 (10.34)	1 (2.85)
10''					
11	1 (1.3)	3 (7.89)		1 (3.44)	1 (2.85)
11'	2 (2.56)	2 (5.26)	2 (18.18)		
12	1 (1.3)		1 (9.09)		
13	2 (2.56)				

a Number of samples in each category.

( ) Percentage

Table 11 (cont'd.)

Distribution of *Pseudomonas aeruginosa* REA
Patterns Among Sanitary Sewer, Priority and Non-Priority Storm Sewer and
Storm Water Runoff Samples Collected during Periods of Wet and Dry Weather

REA Pattern	Sanitary Sewer Sites D, E, F	Priority Storm Sewer Sites A,B,C,	Y	Non-Priority Storm Storm Sewer Sites Wet X, Z	Storm Water Runoff Sites P,Q,G,R
13'	8 (10.25)	5 (13.15)			
14	1 (1.3)	3 (7.89)			1 (2.85)
15		1 (2.63)			
16		2 (5.26)			
17	2 (2.56)				
18				4 (13.79)	6 (17.14)
19				1 (3.44)	
20				2 (6.89)	3 (8.57)
21				1 (3.44)	
22				1 (3.44)	
23				4 (13.79)	
24				1 (3.44)	
25				1 (3.44)	
26				1 (3.44)	
27					1 (2.85)
28					2 (5.71)
29					1 (2.85)
29'				1 (3.44)	
Total	78[a]	38	11	29	35

[a] Number of samples in each category.
( ) Percentage

were found in the storm water runoff samples at site Q, and in the sewer that collected this same storm water at site Z. Similarly, REA pattern 20 isolates were recovered from water runoff at site R and in the receiving storm sewer at site X. The fact that the genotype 20 organisms belonged to both serotypes 1 and 3 suggests that genotyping is probably a more concise method of fingerprinting P. aeruginosa than serotyping. From the results, genotyping appears to be a promising method of tracing P. aeruginosa from human and animal sources.

Similar to the 1986-87 streptococcal results, acid curd production was observed among the sanitary sewage and high priority storm sewage S. faecalis isolates (Table 64 to 69, Appendix). However, there did not appear to be a marked difference between the aforementioned isolates and those from the non-priority storm sewers. All S. faecalis isolates tended to produce a variety of reactions in litmus milk.

Unlike the P. aeruginosa organisms, genotyping of the fecal streptococci did not produce any concise results. One hundred and ninety-two streptococcal isolates, 60 of which were from the sanitary sewer, were genotyped. A total of 64 different REA patterns were identified among the isolates.

Streptococcus faecalis subsp. faecalis (Table 12) was the only species that had the predominant genotypes 5 and 8 occurring in both the sanitary sewer and the priority storm sewer isolates. As stated previously Mundt (1973) has suggested that S. faecalis isolates from human feces will produce an acid curd in litmus milk. However, no relationship between the 5 and 8 pattern isolates and those that produced an acid curd was observed. Although the S. faecium isolates were distributed among 31 different REA patterns, genotype 9 was found in all three sites of the sanitary sewer and genotype 5 was recovered from all sites in the priority storm sewer. S. faecium subsp. casseliflavus (Table 13)

Table 12

Distribution of Streptococcus faecalis subsp. faecalis REA Patterns Among Sanitary Sewer, Priority and Non-Priority Storm Sewer and Storm Water Runoff Samples Collected during Periods of Wet and Dry Weather

REA Pattern	Sanitary Sewer Sites D, E, F	Priority Storm Sewer Sites A,B,C,	Y	Non-Priority Storm Storm Sewer Sites Wet X, Z	Storm Water Runoff Sites P,Q,G,R
1'					1
3					
4		1			
5	1	5			
5'		1			
6	1				1
8	2	5			
8'		1			
9	1				
9'					
10	2		1		
11		1	1		
11'		1			
12'	1				
14	1			1	
16	2				
16''	1			1	
17					
23					
31					
33					
34					
35					
42					
46					
47					
48					
Total	12	15	2	2	2

Table 13
Distribution of Streptococcus faecium subsp. casseliflavus REA Patterns Among Sanitary Sewer, Priority and Non-Priority Storm Sewer and Storm Water Runoff Samples Collected during Periods of Wet and Dry Weather

REA Pattern	Sanitary Sewer Sites D, E, F	Priority Storm Sewer Sites A,B,C, 7	Y	Non-Priority Storm Storm Sewer Sites Wet and dry X, Z	Storm Water Runoff Sites P,Q,G,R
1'					
3		1			
4					
5	1				
5'					
6					
8					1
8'					
9	1				
9'				1	
10	1	2			
10'		1			
11		1	1		
11'					
12	1				1
14					1
16				1	
16'					
17		1			
23		1		1	
31				1	
33				1	
34				2	
35				1	
42					1
46				1	1
47				1	
48				1	
Total	4	7	1	11	5

had 21 different REA patterns that were widely distributed among the isolates from different sources. In general, no relationship between the source of isolation and the genotype could be found among the 192 streptococcal isolates studied.

More insight into the origin of fecal wastes in storm sewer lines was provided by the speciation of fecal streptococci (Tables 59 to 63, Appendix). The results showed that S. faecium tended to be equally represented in all sample categories. On the other hand, S. faecalis subsp. faecalis (Fig. 7) was found more frequently in sanitary and priority storm sewers than in surface runoff and non-priority sewers. In contrast, S. faeccium subsp. casseliflavus (Fig. 8) predominated in non-priority storm sewer water and was notably evident in storm water runoff. The organism was virtually nonexistent in sanitary sewage. Levels of S. faecium subs. casseliflavus in priority storm sewers were intermediate between the counts found in sanitary sewage and in the non-priority storm sewer. These results concur with our previous data (Seyfried, Harris, Young, 1986 unpublished) which showed that S. faecium subsp. casseliflavus could be isolated from animal feces but not from human fecal specimens.

Summary of 1988 Survey Results

1. The levels of fecal coliforms, Escherichia coli, P. aeruginosa and Bifidobacterium sp. suggest that there is an impact near site A in the storm sewer line that may be due to human fecal pollution.
2. While Bifidobacterium breve and B. adolescentis are found in human feces, they cannot be used to differentiate human from animal sources of pollution because they can be isolated from animals such as dogs and chickens.

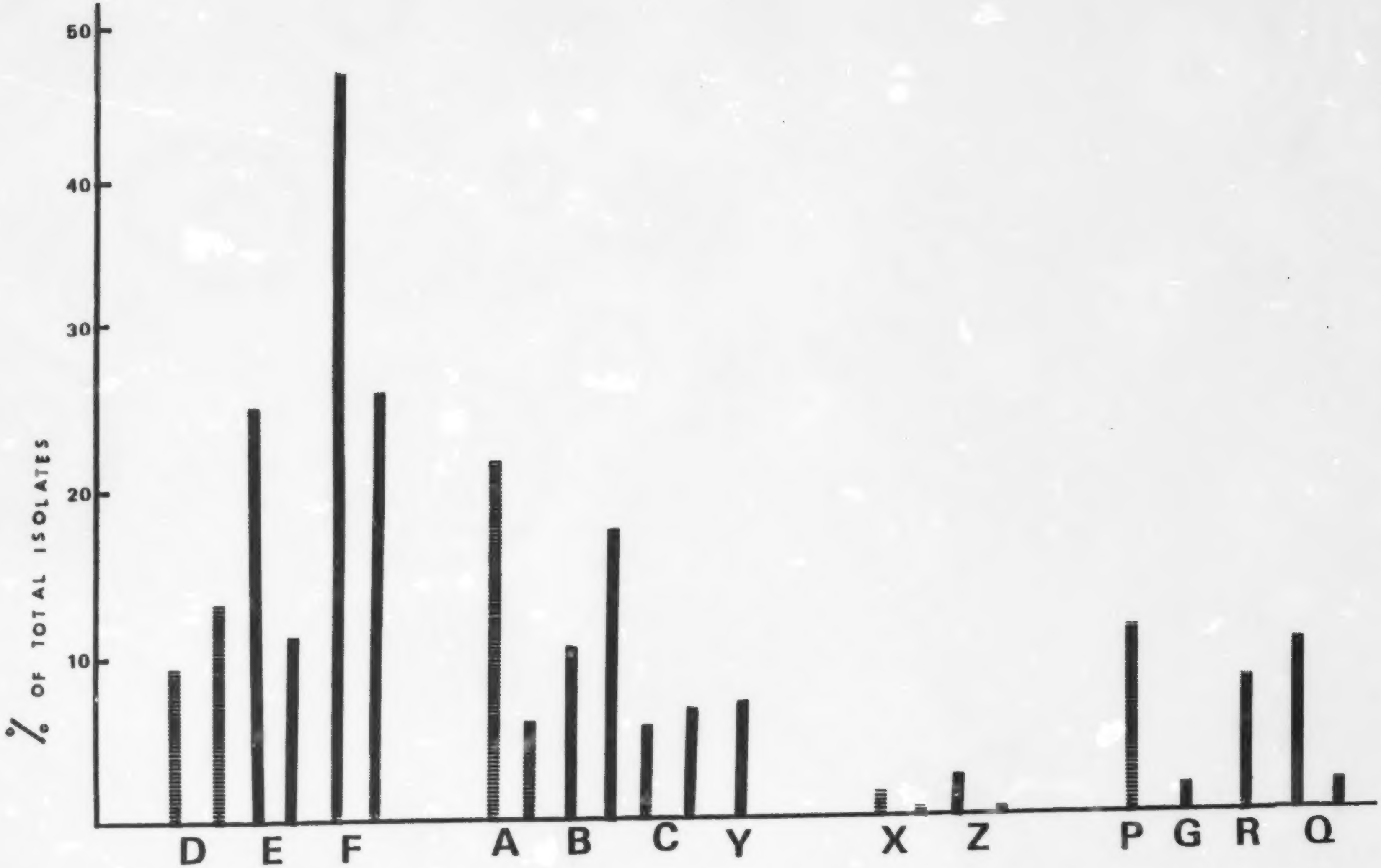

Fig. 7 Percentage distribution of Streptococcus faecalis subsp. faecalis among the sanitary sewage sites (D,E,F), the priority storm sewer (A,B,C,Y), the non-priority storm sewer (X,Z) and the storm water runoff (P,G,R,Q) locations.

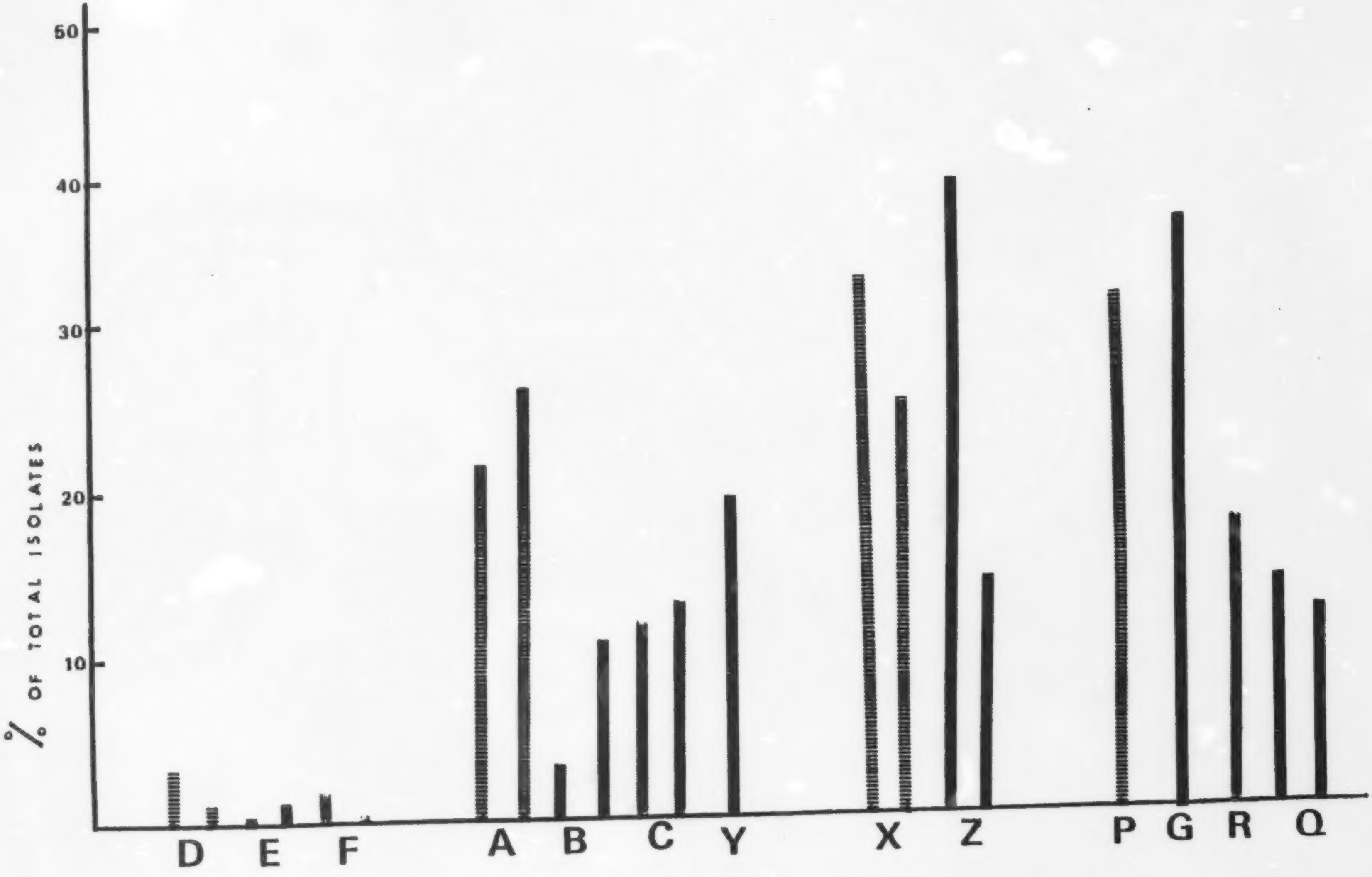

Fig. 8 Percentage distribution of Streptococcus faecium subsp. casseliflavus among the sanitary sewage sites (D,E,F), the priority storm sewer (A,B,C,Y), the non-priority storm sewer (X,Z), and the storm water runoff (P,G,R,Q) locations.

3. Genotyping of *Bifidobacterium* isolates may provide a more precise method of source differentiation, but further work has to be done to find a suitable genotyping method.
4. *P. aeruginosa* genotyping may be of value in tracing sources of pollution. Serotyping, however does not provide results that are specific enough.
5. Genotyping of fecal streptococci is not recommended as a method of source determination since the wide variety of patterns produced yield inconclusive results.
6. Speciating fecal streptococci is a useful means of characterizing sewer or storm water content. For example, *S. faecalis* subs. *faecalis* is found predominantly in sanitary and priority storm sewers whereas *S. faecium* subsp. *casseliflavus* has not been isolated from human feces.

DISCUSSION OF ADDITIONAL PROJECTS

A. Comparative Study of the Survival of Indicator Bacterial Species by Eric Bauer (see Appendix)

The complete project is outline in the Appendix. The study had two objectives. One was to compare the die-off rates of *E. coli*, *P. aeruginosa*, *S. faecalis* var. *faecalis*, *S. faecium* and *B. longum* at room temperature and at 15° C. The second was to examine the lethality of chlorine on *E. coli*, *P. aeruginosa*, *S. faecium* var. *casseliflavus* and *B. breve*.

The results of the project showed that *B. longum* died off more quickly than the other organisms at room temperature. However, at 15° C, the usual temperature in a storm sewer, *P. aeruginosa*, *E. coli*, and *B. longum* all had a shorter life span than the streptococci. Thus these three organisms would be better indicators of recent pollution in storm sewers than fecal streptococci.

Of the organisms tested, *B. breve* appeared to be the most sensitive to chlorine treatment. The combined characteristics of sensitivity to adverse conditions and ability to survive for only a relatively short period of time outside the body support the proposal that Bifidobacterium species would be useful indicators of fresh fecal contamination.

B. The Isolation and Identification of Bifidobacteria from Fecal and Sewage Samples by In-ja Huh (see Appendix)

An attempt was made in this study to find an optimal medium for the recovery of bifidobacteria from human and animal feces and from sewage. A comparison was also made with the HBSA medium designed to isolate sorbitol-fermenting species of bifidobacteria. (In HBSA, lactose is replaced by sorbitol).

The results showed that the YN-17 medium yielded fewer false positive target colonies than HBSA (5% and 10%, respectively). The majority of false positive targets were found to be fecal streptococci. Lithium chloride, in concentrations of 0.3% and 0.4%, was added to YN-17 and HBSA to inhibit streptococcal growth; however, it was not successful in reducing the contaminant levels in these media.

The YN-17 medium was developed by Mara and Oragui in 1983. It was found in this study that colonies the authors described as bifidobacteria (i.e. blue centre with pale green periphery) were, in fact, a mixture of bifidobacteria and streptococci. The characterization profiles indicated that the smaller dark green/blue flatter colonies were bifidobacteria and those exhibiting a pale green periphery were contaminating streptococci or, more frequently, a mixture of the two organisms. Any colonies that were pale green in colour and did not contain a darker centre were found to be streptococci.

Sorbitol-fermenting (B. breve) species were isolated from human and cat feces on the HBSA medium. It should be noted however that none of the cat isolates were able to ferment sorbitol in subsequent tube tests.

Bifidobacterium adolescentis was the most common species of bifidobacteria in sewage and human fecal samples. This organism was not found in cats.

The data showed that compared with E. coli levels, bifidobacteria counts were 100 times greater in feces and 10 times greater in sewage samples.

C. A Study of the Survival of Bifidobacteria and their Role in Water Quality Control by Sheila Shibata (see Appendix).

The main objectives of this research project were to determine the most suitable medium for the isolation of bifidobacteria from feces and from environmental samples and to compare the in vitro survival characteristics of bifidobacteria and E. coli. The results of the assessment of YN-17, MRS and MPN media showed that the MRS medium recovered not only Bifidobacterium sp. but high levels of lactobacilli and fecal streptococci as well. YN-17 and MPN were equally selective for bifidobacteria but YN-17 had an advantage in that it required a 24 h shorter incubation period. Bifidobacteria were found to exist in feces at a 1000-fold higher concentration than E. coli. The sorbitol-fermenting bifidobacteria species, however, were present in feces in numbers comparable with E. coli (i.e. 10^7).

When bifidobacteria and E. coli were added to dialysis diffusion chambers and suspended in Lake Ontario water it was found that Bifidobacterium sp. died off within 24 h of exposure whereas E. coli died off more slowly and could still be isolated after 48 h or more.

Since bifidobacteria are found in high levels in feces and have a tendency to die off rapidly in the environment these organisms show promise as indicators of recent human fecal pollution.

D. Clostridium perfringens and Bifidobacterium sp. as Tracers in Storm Sewers by Eric Hani (see Appendix)

This project examined the feasibility of using C. perfringens and Bifidobacterium sp. as indicators of fecal pollution from warm-blooded animals. The data showed that both indicators were present in high concentrations in feces but that bifidobacteria could be isolated on a more consistent basis. The graphs indicated that bifidobacteria levels were high in both sanitary and high priority storm sewage. Clostridium perfringens densities, on the other hand, were comparatively low in the high priority storm sewage. The histogram giving the geometric mean concentrations of E. coli, C. perfringens and bifidobacteria showed that levels of all three indicators tended to be higher in the sanitary and high priority storm sewage samples in comparison with the non-priority storm sewer samples. Clostridium perfringens could not be recovered from one non-priority storm sewer sample. There did not appear to be any major trends with respect to source when the ratios of the three indicator organisms were compared (Table E-1).

In a random sampling of human, cat and dog feces, bifidobacteria levels were found to be higher than E. coli in humans. Although C. perfringens was recovered from the cat fecal sample, no bifidobacteria were found. As previously mentioned, Mara and Oragui (1983) found sorbitol fermenting species of Bifidobacterium to be exclusive to human feces. However, this reaction does not appear to be a practical criterion for source tracing since only a small percentage of the sanitary sewage isolates were sorbitol fermenters.

E. Characterizations of Pseudomonas aeruginosa from Storm and Sanitary Sewers by Rita Harmandayan (see Appendix)

This project was designed to investigate the use of serotyping and genotyping to distinguish P. aeruginosa strains from human and non-human sources. The data showed that levels of P. aeruginosa were close to 1 x 10^4 in sanitary sewage and 1 x 10^1 in storm sewage samples. Approximately equal numbers of isolates from both sources were serotyped using the Difco serotyping kit number 3081-32 (Pseudomonas aeruginosa antigen kit). Serotype 0:6 was the most frequently indentified serotype in both the storm and sanitary sewers. Serotypes 0:1 and 0:11 were also commonly isolated from both sources. Serotype 0:16 was recovered from storm sewers only, with a frequency of 5.4%. Serotype 0:10, on the other hand, was isolated solely from sanitary sewers with a frequency of 21.9%. It is possible, therefore, that the presence of type 0:10 may be indicative of human fecal contamination.

To determine if identical genotype patterns were present within each serogroup, restriction enzyme analysis was performed on isolates with common serotypes. Of the six different restriction enzymes used to cut the genomic DNA, Sma I produced the best banding pattern on polyacrylamide gels. The results showed that not all isolates with the same serotype possessed the same genome pattern. This is explained by the fact that the genes responsible for serotyping the 0-Ag comprise a small percentage of the total genome and that other genes besides those that code for the 0-Ag will appear when the total chromosomal DNA is compared. Thus, the REA technique was shown in this study to be highly specific; it further subdivides one serotype into different genotypes. In future, a probe could be developed from either total chromosomal DNA or a specific fragment common to human strains that would allow hybridization against other P. aeruginosa strains. The probe would help to differentiate between isolates from human and non-human fecal waste.

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APPENDIX A

Table 14

Sampling Dates for Dry and Wet Weather Surveys

Year	Date	Category
1986	October 21, 22 and 28, November 18	Dry Weather I
1987	June 10, 11, 12 and 19	Dry Weather II
	August 17	Dry Weather
1988	June 20, 21, 28	Dry Weather
	August 15, 22	Dry Weather
1987	July 14	Wet Weather I
	September 18	Wet Weather
	October 21	Wet Weather

Table 15

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, *ESCHERICHIA COLI*, FECAL STREPTOCOCCI, ENTEROCOCCI AND *PSEUDOMONAS AERUGINOSA* RECOVERED FROM HIGH PRIORITY STORM SEWAGE DURING DRY WEATHER SURVEY 1, 1986

DATE/SAMPLE	Parameter per 100 mL of Sample							
	Fecal coliforms (m-Tec)	*E. coli* (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	*Pseudomonas aeruginosa* (m-PA)	Bifidobacterium (YN-17)	*Clostridium perfringens* (m-CP2)
October 21								
A	2.8×10^3	2.3×10^3	3.5×10^2	5.3×10^2	8.0	5.25		
B	1.07×10^4	7.6×10^3	1.1×10^4	4.4×10^4	1.0	9.6×10^1		
C	8.3×10^3	3.9×10^3	9.8×10^3	4.2×10^3	0.85	1.6×10^1		
October 22								
A	3.7×10^3	3.0×10^3	5.5×10^2	8.3×10^2	6.73	2.6×10^1		
B	3.0×10^3	1.8×10^3	3.7×10^2	6.3×10^2	8.1	4.3×10^1		
C	5.0×10^2	3.4×10^2	1.4×10^2	7.6×10^1	3.6	7.24		
October 28 Rainfall A	1.6×10^4	1.5×10^4	2.7×10^3	1.8×10^3	6.0	< 2		
Previous B	2.14×10^4	1.45×10^4	1.95×10^3	1.74×10^3	11.0	< 2		
Evening C	3.9×10^4	2.45×10^4	2.63×10^4	2.6×10^4	1.5	< 2		
November 18								
A	6.02×10^4	5.4×10^4	3.0×10^4	2.0×10^4	2.0	8.3×10^1		
B	2.63×10^4	2.34×10^4	8.91×10^2	3.6×10^2	29.5	3.0×10^1		
C	1.3×10^3	7.4×10^2	7.1×10^2	5.13×10^2	1.8	2.0*		

* Approximate data.
No data available for Bifidobacteria, *Clostridium perfringens*.

Table 16

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI AND PSEUDOMONAS AERUGINOSA RECOVERED FROM SANITARY SEWAGE DURING DRY WEATHER SURVEY 1, 1986

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	E. coli (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	Pseudomonas aeruginosa (m-PA)	Bifidobacterium (YN-17)	Clostridium perfringens (m-CP2)
October 21								
D	2.57×10^6	1.7×10^6	5.6×10^5	6.5×10^5	4.6	6.9×10^4		
E	1.9×10^6	9.6×10^5	1.2×10^5	3.5×10^4	15.8	1.8×10^4		
F	4.3×10^6	2.0×10^6	3.9×10^5	4.0×10^5	11.0	1.0×10^4		
October 22								
D	2.5×10^6	2.2×10^6	4.1×10^4	5.13×10^4	61.0	1.1×10^5		
E	6.2×10^6	2.2×10^6	4.2×10^5	4.27×10^5	14.8	4.4×10^3		
F	4.0×10^6	1.9×10^6	8.9×10^5	9.1×10^5	4.5	4.2×10^3		
October 28								
Rainfall D	1.07×10^6	6.2×10^5	8.32×10^4	7.76×10^4	12.9	$< 1.0 \times 10^2$		
Previous E	1.3×10^6	7.2×10^5	1.12×10^5	1.23×10^5	11.6	$< 1.0 \times 10^2$		
Evening F	2.3×10^6	1.5×10^6	4.5×10^5	5.62×10^5	5.1	$< 1.0 \times 10^2$		
November 18								
D	2.3×10^6	1.7×10^6	3.0×10^5	2.8×10^5	7.7	7.1×10^3		
E	3.63×10^6	2.8×10^6	5.8×10^4	4.57×10^4	62.6	5.4×10^4		
F	6.5×10^6	4.9×10^6	1.3×10^5	2.95×10^5	50.0	1.2×10^4*		

* Approximate value.
No Data available for Bifidobacterium, Clostridium perfringens.

Table 17

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM HIGH PRIORITY AND NON-PRIORITY STORM SEWAGE DURING DRY WEATHER SURVEY 2, 1987

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	*E. coli* (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	*Pseudomonas aeruginosa* (m-PA)	Bifidobacterium (YN-17)	*Clostridium perfringens* (m-CP2)
June 10								
Non-priority X	4.07×10^{3}	7.6×10^{2}	6.90×10^{3}	1.12×10^{3}	0.59	8.5	< 0	1.12×10^{2}
High priority storm A	4.5×10^{4}	4.0×10^{4}	2.9×10^{4}	4.9×10^{3}	1.55	1.3×10^{2}	2.0×10^{4}	3.2×10^{2}
B	4.9×10^{4}	4.8×10^{4}	3.4×10^{3}	3.5×10^{2}	14.4	3.1×10^{2}	1.8×10^{5}	4.6×10^{1}
C	7.2×10^{3}	4.8×10^{3}	8.1×10^{4}	2.4×10^{3}	0.09	1.1×10^{2}	2.6×10^{2}	1.2×10^{2}
Y	1.26×10^{3}	1.0×10^{3}	1.8×10^{3}	5.1×10^{2}	0.7	2.0×10^{1}	3.7×10^{3}	6.2×10^{2}
June 11								
Non-priority X	5.9×10^{2}	4.1×10^{2}	1.0×10^{3}	1.2×10^{3}	0.6	2.3	10.0	1.3×10^{2}
High priority A	5.6×10^{4}	5.5×10^{4}	2.8×10^{3}	4.8×10^{2}	20.0	4.9×10^{1}	1.5×10^{5}*	1.3×10^{3}
B	6.8×10^{4}	6.8×10^{4}	9.3×10^{3}	2.2×10^{3}	7.3	2.6×10^{2}	1.3×10^{5}*	4.3×10^{3}
C	8.5×10^{4}	7.9×10^{4}	4.8×10^{4}	1.2×10^{5}	1.8	2.2×10^{2}	8.5×10^{4}*	4.7×10^{3}
Y	2.6×10^{3}	2.1×10^{3}	3.5×10^{3}	6.5×10^{2}	0.7	1.2×10^{1}	4.1×10^{4}	6.5×10^{2}
June 12								
Non-priority X	5.8×10^{3}	4.5×10^{3}	1.05×10^{4}	7.4×10^{3}	0.55	7.6×10^{1}	NA	NA
High priority A	4.3×10^{4}	2.3×10^{4}	2.3×10^{4}	2.5×10^{4}	1.9	2.8×10^{2}	NA	NA
B	1.1×10^{5}	3.7×10^{4}	1.6×10^{4}	1.9×10^{4}	6.9	3.1×10^{3}	NA	NA
C	1.1×10^{4}	4.8×10^{3}	1.8×10^{4}	8.1×10^{3}	0.69	6.6×10^{1}	NA	NA
Y	2.3×10^{4}	1.6×10^{4}	1.4×10^{4}	2.6×10^{4}	1.64	4.1×10^{2}	NA	NA

* Approximate data

NA - Not analyzed due to insufficient sample volume.

Table 18

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM SANITARY SEWAGE DURING DRY WEATHER SURVEY 2, 1987

Parameter per 100 mL of Sample

DATE/SAMPLE		Fecal coliforms (m-Tec)	E. coli (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	Pseudomonas aeruginosa (m-PA)	Bifidobacterium (YN-17)	Clostridium perfringens (m-CP2)
June 10									
	D	2.2×10^6	2.2×10^6	5.2×10^4	2.9×10^4	42.3	1.7×10^4	3.0×10^3	2.2×10^4
	E	6.0×10^6	3.6×10^6	2.0×10^5	3.3×10^5	30.0	1.7×10^4	1.3×10^4	1.9×10^5
	F	1.7×10^6	1.3×10^6	1.1×10^5	1.1×10^5	15.5	1.7×10^4	NA	3.3×10^4
June 11									
	D	2.0×10^6	1.9×10^6	2.9×10^5	2.1×10^5	500.0	9.5×10^3	2.2×10^5	2.5×10^4
	E	2.95×10^6	2.6×10^6	6.6×10^4	6.8×10^4	44.3	2.0×10^4	2.0×10^4	1.4×10^4
	F	3.5×10^6	3.4×10^6	2.0×10^5	1.9×10^5	17.5	5.9×10^3	3.0×10^4	2.2×10^4
June 12									
	D	1.3×10^6	1.3×10^6	1.7×10^4	2.0×10^4	76.5	6.9×10^3	NA	NA
	E	3.0×10^6	1.1×10^6	1.05×10^5	5.9×10^4	28.6	1.8×10^4	NA	NA
	F	6.8×10^5	5.8×10^5	9.5×10^5*	6.8×10^5*	0.72	6.0×10^3	NA	NA

* Approximate data

NA - Not analyzed due to insufficient sample volume.

Table 19

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM HIGH PRIORITY AND NON-PRIORITY STORM SEWAGE DURING DRY WEATHER SURVEY 2, 1987

Parameter per 100 mL of Sample

DATE/SAMPLE		Fecal coliforms (m-Tec)	E. coli (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	Pseudomonas aeruginosa (m-PA)	Bifidobacterium (YN-17)	Clostridium perfringens (m-CP2)
June 19									
Non priority	X	1.1×10^3	4.6×10^2	2.3×10^3	NA	0.5	1.4*	1.7×10^1	NA
High priority	A	5.2×10^4	1.9×10^4	5.6×10^3	NA	9.3	3.8×10^2	6.0×10^4	NA
	B	1.4×10^5	1.2×10^5	7.9×10^3	NA	17.7	6.6×10^2	1.3×10^5	NA
	C	1.1×10^3	7.2×10^2	7.6×10^4	NA	0.01	5.0×10^1	1.4×10^2	NA
	Y	2.7×10^3	1.95×10^3	2.9×10^3	NA	0.93	2.2×10^1	1.1×10^4	NA
August 17									
High priority	A	1.1×10^5	8.9×10^4	1.32×10^5	NA	0.83	1.4×10^2	7.8×10^3	4.1×10^2*
	B	2.6×10^4	1.91×10^4	1.6×10^4	NA	1.63	4.7×10^2	9.1×10^3	9.1×10^1
	C	4.7×10^3	1.6×10^3	2.0×10^4	NA	0.24	7.41×10^1	4.0×10^1	1.0×10^2
	Y	1.26×10^5	7.6×10^4	4.9×10^3	NA	25.7	3.8×10^2	NA	8.32×10^1

* Clostridium perfringens samples were heat-treated at 70° C for 30 minutes.

NA - Not analyzed due to insufficient sample volume.

Table 20

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM SANITARY SEWAGE DURING DRY WEATHER SURVEY 2, 1987

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	E. coli (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	Pseudomonas aeruginosa (m-PA)	Bifidobacterium (YN-17)	Clostridium perfringens (m-CP2)
June 19								
D	2.6×10^6	2.4×10^6					7.8×10^4	
E	2.4×10^6	2.4×10^6					9.8×10^5	
F	1.9×10^6	1.7×10^6					1.7×10^5	
August 17								
D	2.9×10^6	2.0×10^6	2.1×10^5	NA	13.8	1.35×10^4	6.0×10^4	1.0×10^2
E	2.5×10^7	1.3×10^7	7.94×10^4	NA	314.9	6.5×10^4	1.0×10^3	1.0×10^2
F	1.9×10^7	1.82×10^7	1.1×10^6	NA	17.27	2.3×10^4	4.0×10^6	1.0×10^2

* Clostridium perfringens samples were heat-treated at 70° C for 30 minutes.

NA - Not analyzed due to insufficient sample volume.

Table 21

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM NON-PRIORITY STORM SEWAGE AND RUN-OFFS DURING WET WEATHER SURVEYS IN 1987

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	*E. coli* (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	*Pseudomonas aeruginosa* (m-PA)	Bifidobacterium (YN-17)	*Clostridium perfringens* (m-CP2)	
								15 min.	30 min.
July 14									
Non-priority X	1.32×10^5	8.5×10^4	8.9×10^4	9.8×10^4	1.48	1.45×10^3	2.4×10^3	1.0×10^3	1.29×10^3
Y	1.35×10^5	2.24×10^4	5.25×10^5		0.26	1.23×10^4	1.86×10^3	3.8×10^2	5.4×10^2
Z	4.07×10^4	1.1×10^4	1.2×10^5	7.24×10^4	0.34	5.01×10^2	4.37×10^2	NA	
Run-offs P	5.75×10^3	4.8×10^3	2.5×10^4	NA	0.23	5.6×10^2		NA	
Q	6.34×10^3	2.9×10^3	3.02×10^3	NA	2.10	4.6×10^1		NA	
September 18									
Non-priority X	7.24×10^4	4.68×10^4	1.62×10^5	5.89×10^4	0.45	2.09×10^3		NA	
Run-offs G	1.29×10^5	7.59×10^4	2.04×10^5	5.89×10^4	0.63	2.63×10^3		NA	
October 21									
Non-priority X	1.35×10^3	1.18×10^3	2.34×10^4	NA	0.06	1.91×10^2	7.41×10^2	NA	
Z	4.27×10^4	4.17×10^4	1.29×10^5	NA	0.33	1.62×10^2	3.63×10^2	NA	
Run-offs R	3.89×10^4	3.80×10^4	NA	NA		1.29×10^3	6.03×10^2	NA	
Q	6.03×10^3	5.25×10^3	5.62×10^4	NA	0.11	1.32×10^2	3.98×10^3	NA	

Table 22

A COMPARISON OF FECAL COLIFORM/FECAL STREPTOCOCCUS (FC/FS) RATIOS

Sample Date	FC:FS Ratio at Sampling Sites												
	X	Y	Z	P	Q	R	G	A	B	C	D	E	F
Dry weather													
1986													
Oct. 21								8.0	1.0	0.85	4.6	15.8	11.0
Oct. 22								6.73	8.1	3.6	61.0	14.8	4.5
Oct. 28								6.0	11.0	1.5	12.9	11.6	5.1
Nov. 18								2.0	29.5	1.8	7.7	62.6	50.0
1987													
June 10		0.7						1.55	14.4	0.09	42.3	30.0	15.5
June 11	0.6	0.7						20.0	7.3	1.8	500.0	44.3	17.5
June 12	0.55	1.64						1.9	6.9	0.69	76.5	28.6	0.72
June 19	0.5	0.93						9.3	17.7	0.01			
Aug. 17		25.7						0.83	1.63	0.24	13.8	314.9	17.27
1988													
June 20		0.46						6.6	1.18	2.24	5.61	5252.6	2.19
June 21		0.23						2.19	0.57	0.06			
June 28	0.02		0.03										
Aug. 15		0.85						1.65		6153.8	74.1		5.6
Aug. 22	257.6	41.8	88.9					5.13	5.0	0.68	0.07	664.6	6.18
Wet Weather													
1987													
July 14	1.48	0.26	0.34	0.23	2.10								
Sept. 18	0.45						0.63						
Oct. 21	0.06		0.33		0.11	NA							

Table 23

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather Survey 1, Oct. 21, 1986

Species and variant	High Priority			Sanitary Sewage		
	A [52]	B [31]	C [46]	D [44]	E [38]	F [53]
S. faecalis var						
faecalis	11 (21.2)			-	1 (2.6)	15(28.3)
liquefaciens	1 (1.9)	6 (19.4)		18 (40.9)		6 (11.3)
zymogenes	-	7 (22.6)			1 (2.6)	1 (1.9)
S. faecium	20 (38.5)	10 (32.3)	25 (54.3)	19 (43.2)	15 (39.5)	3 (5.7)
S. faecium var. casseliflavus	12 (23.1)		4 (8.7)	3 (6.8)		3 (5.7)
S. durans	3 (5.8)		1 (2.2)	4 (9.1)	21 (55.3)	25 (47.2)
S. avium	2 (3.8)	2 (6.5)	1 (2.2)			
S. bovis	2 (3.8)	3 (9.7)				
not FS			6 (13.0)			
FS	1 (1.9)	1 (3.2)				
S. lactis		2 (6.5)	9 (19.6)			

( ) percentage
[ ] total isolates identified from source

Table 24

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather Survey 2, Oct. 22, 1986

Species and variant	High Priority			Sanitary Sewage		
	A [52]	B [42]	C [40]	D [46]	E [39]	F [37]
S. faecalis var.						
faecalis	5 (9.6)	2 (4.8)	2 (5.0)	5 (10.9)	22 (56.4)	31 (83.8)
liquefaciens	4 (7.7)	9 (21.4)	5 (12.5)			
zymogenes	1 (1.9)	-				
S. faecium	15 (28.8)	22 (52.4)	12 (30.0)	31 (67.4)	17 (43.6)	5 (13.5)
S. faecium var. casseliflavus	11 (21.2)	2 (4.8)	13 (32.5)	1 (2.2)		
S. durans	11 (21.2)	3 (7.1)	1 (2.5)	8 (17.4)		1 (2.7)
S. avium	5 (9.6)	3 (7.1)	3 (7.5)			
S. bovis						
not FS			1 (2.5)	1 (2.2)		
FS		1 (2.4)	3 (7.5)			

( ) percentage
[ ] total isolates identified from source

Table 25

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather Survey 1, Oct. 28, 1986

Species and variant	High Priority			Sanitary Sewage		
	A [18]	B [20]	C [20]	D [19]	E [18]	F [16]
S. faecalis var.						
faecalis		1 (5.0)	1 (5.0)	2 (10.5)	4 (22.2)	3 (18.8)
liquefaciens				1 (5.3)		1 (6.3)
zymogenes						1 (6.3)
S. faecium	17 (94.4)	18 (90.0)	18 (90.0)	11 (57.9)	13 (72.2)	11 (68.8)
S. faecium var. casseliflavus	1 (5.6)	1 (5.0)				
S. durans			1 (5.0)	5 (26.3)	1 (5.6)	
S. avium						
S. bovis						
not FS						
FS						

( ) percentage
[ ] total isolates identified from source

Table 26

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather Survey 1, Nov. 18, 1986

Species and variant	High Priority			Sanitary Sewage		
	A [36]	B [32]	C [27]	D [10]	E [7]	F [10]
S. faecalis var.						
faecalis	12 (33.3)	5 (15.6)	4 (14.8)	2 (20.0)	1 (14.3)	5 (50.0)
liquefaciens	1 (2.8)	-	7 (25.9)	-	1 (14.3)	-
zymogenes		1 (3.1)				
S. faecium		6 (18.8)	10 (37.0)	7 (70.0)	5 (71.4)	5 (50.0)
S. faecium var. casseliflavus	15 (41.7)	2 (6.2)		1 (10.0)	-	-
S. durans	1 (2.8)	2 (6.2)	5 (18.5)			
S. avium	5 (13.9)	10 (31.3)				
S. bovis	1 (2.8)	4 (12.4)	1 (3.7)			
not FS		1 (3.1)				
FS	1 (2.8)	1 (3.1)				

( ) percentage
[ ] total isolates identified from source

Table 27

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather June 10, 1987

Species and variant	High Priority			Non-Priority		Sanitary Sewage		
	A [60]	B [57]	C [56]	X [56]	Y [56]	D [81]	E [87]	F [67]
S. faecalis var.								
faecalis	2 (3.3)	25 (43.9)	1 (1.8)	3 (5.4)	12 (21.4)	13 (16.1)	9 (10.4)	17 (25.4)
liquefaciens	3 (5.0)	4 (7.0)	10 (17.9)	4 (7.1)	10 (17.9)	14 (17.3)	15 (17.2)	28 (41.8)
zymogenes	-	-	1 (1.8)	-	1 (1.8)	-	-	2 (3.0)
S. faecium	29 (48.3)	6 (10.5)	6 (10.7)	20 (35.7)	13 (23.2)	44 (54.3)	57 (65.5)	18 (26.9)
S. faecium var. casseliflavus	16 (26.7)	6 (10.5)	7 (12.5)	26 (46.4)	9 (16.1)	4 (4.9)	3 (3.5)	1 (1.5)
S. durans	2 (3.3)	2 (3.5)	3 (5.4)	2 (3.6)	3 (5.4)	5 (6.2)	1 (1.2)	-
S. avium	3 (5.0)	8 (14.0)	5 (8.9)	-	2 (3.6)	-	-	-
S. bovis	-	3 (5.3)	-	-	2 (3.6)			
not FS	2 (3.3)		7 (12.5)	1 (1.8)	1 (1.8)	1 (1.2)		
FS	2 (3.3)	1 (1.8)	9 (16.1)				2 (2.3)	
S. lactis	1 (1.7)	2 (3.5)	6 (10.7)		2 (3.6)			
S. equinus	-	-	-	-	1 (1.8)	-	-	-
S. galactis			1 (1.8)					1 (1.5)

( ) percentage of total
[ ] number of isolates identified

Table 28

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather June 11, 1987

Species and variant	High Priority			Non-Priority		Sanitary Sewage		
	A [65]	B [63]	C [68]	X [62]	Y [58]	D [59]	E [57]	F [39]
S. faecalis var.								
faecalis	9 (13.9)	16 (25.4)	14 (20.6)	2 (3.2)	3 (5.2)	6 (10.2)	3 (5.3)	-
liquefaciens	3 (4.6)	8 (12.7)	8 (11.8)	-	11 (19.0)	6 (10.2)	17 (29.8)	-
zymogenes	3 (4.6)	-	-	1 (1.6)	6 (10.4)	-	9 (15.8)	-
S. faecium	7 (10.8)	26 (41.3)	26 (38.2)	14 (22.6)	23 (39.7)	41 (69.5)	28 (49.1)	39(100.0)
S. faecium var.								
casseliflavus	15 (23.1)	6 (9.5)	9 (13.2)	34 (54.8)	14 (24.1)	-	-	-
S. durans	1 (1.5)	2 (3.2)	5 (7.4)	2 (3.2)	1 (1.7)	4 (6.8)	-	-
S. avium	11 (16.9)	3 (4.8)	4 (5.9)	1 (1.6)	-	2 (3.4)	-	-
S. bovis	8 (12.3)	-	1 (1.5)	-	-	-	-	-
not FS	5 (7.7)	2 (3.2)	-	2 (3.2)	-	-	-	-
FS	3 (4.6)	-	1 (1.5)	6 (9.7)	-	-	-	-
S. lactis								
S. equinus								
S. galactis								

( ) percentage of total
[ ] number of isolates identified

Table 29

Fecal Streptococci Populations Recovered from Storm Sewers
and Run-offs during Wet Weather Survey 1, July 14, 1987

Species and variant	Non-Priority		Run-offs	
	X [75]	Z [45]	P [50]	Q [53]
S. faecalis var.				
faecalis	3 (4.0)	1 (2.2)	5 (10.0)	8 (15.1)
liquefaciens	13 (17.3)	11 (24.4)	5 (10.0)	13 (24.5)
zymogenes	2 (2.7)			
S. faecium	12 (16.0)	9 (20.0)	20 (40.0)	15 (28.3)
S. faecium var. casseliflavus	27 (36.0)	17 (37.8)	14 (28.0)	5 (9.4)
S. durans	16 (21.3)	6 (13.3)	1 (2.0)	12 (22.6)
not FS	1 (1.3)			
FS			5 (10.0)	
S. avium	1 (1.3)	1 (2.2)		

( ) percentage of total
[ ] number of isolates identified
P-storm water run-off Parliament & Carlton

Table 30

Fecal Streptococci Populations Recovered from Non-Priority Storm Sewage and Run-offs during Wet Weather Survey, September 18, 1987

Species and variant	Non-Priority	Run-off
	X [42]	G [63]
S. faecalis var.		
faecalis		1 (1.6)
liquefaciens	1 (2.4)	1 (1.6)
zymogenes	1 (2.4)	8 (12.7)
S. faecium	10 (23.8)	23 (36.5)
S. faecium var. casseliflavus	23 (54.8)	22 (34.9)
S. durans	7 (16.7)	7 (11.1)
not FS		1 (1.6)

( ) percentage of total
[ ] number of isolates identified

Table 31

Fecal Streptococci Populations Recovered from Storm Sewers (Non-Priority) and Run-offs during Wet Weather Survey October 21, 1987

Species and variant	Non-Priority		Run-offs	
	X [52]	Z [108]	Q [96]	R [76]
S. faecalis var				
faecalis			1 (1.0)	8 (10.5)
liquefaciens	1 (1.9)		2 (2.1)	3 (3.9)
zymogenes	1 (1.9)		19 (19.8)	1 (1.3)
S. faecium	19 (36.5)	32 (29.6)	53 (55.2)	45 (59.2)
S. faecium var. casseliflavus	21 (40.4)	32 (29.6)	12 (12.5)	13 (17.1)
S. durans	9 (17.3)	44 (40.7)	8 (8.3)	6 (7.9)
not FS	1 (1.9)		1 (1.0)	

( ) percentage of total
[ ] number of isolates identified

Table 32

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather Survey 1, Oct. 21, 1986

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein- ization	Digestion	No Reaction
High priority							
A	12				1 (8.3)		11 (91.7)
B	13		1 (7.7)		6 (46.2)		6 (46.2)
C	-						
Sanitary sewage							
D	18	17 (94.4)		1 (5.6)			
E	2	1 (50.0)		1 (50.0)			
F	22	14 (63.6)		5 (22.7)	2 (9.1)		1 (4.5)

( ) percentage

Table 33

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather Survey 1, Oct. 22, 1986

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High priority							
A	10	2 (20.0)	2 (20.0)		2 (20.0)		4 (40.0)
B	11		4 (36.4)		6 (54.5)		1 (9.1)
C	7				5 (71.4)		2 (28.6)
Sanitary sewage							
D	5	3 (60.0)					2 (40.0)
E	22	17 (77.3)	1 (4.5)				4 (18.2)
F	31	30 (96.8)					1 (3.2)

( ) percentage

Table 34

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather Survey 1, Oct. 28, 1986

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High priority							
A	-						
B	1	1 (100.0)					
C	1		1 (100.0)				
Sanitary sewage							
D	3	2 (66.7)					1 (33.3)
E	4	4 (100.0)					
F	5	3 (60.0)	2 (40.0)				

( ) percentage

Table 35

Percentage of *S. faecalis* Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather Survey 1, Nov. 18, 1986

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Proteinization	Digestion	No Reaction
High priority							
A	13	11 (84.6)			1 (7.7)		1 (7.7)
B	6	5 (83.3)					1 (16.7)
C	11	9 (81.8)		1 (9.1)	1 (9.1)		
Sanitary sewage							
D	2	2(100.0)					
E	2	1 (50.0)		1 (50.0)			
F	5	3 (60.0)					2 (40.0)

( ) percentage

Table 36

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather June 10, 1987

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High priority							
A	5	3 (60.0)	1 (20.0)		1 (20.0)		
B	29	28 (96.6)					1 (3.5)
C	12	2 (16.7)		2 (16.7)	7 (58.3)		1 (8.3)
Non-priority							
X	7	2 (28.6)			3 (42.9)		2 (28.6)
Y	23	18 (78.3)			4 (17.4)		1 (4.4)
Sanitary sewage							
D	27	8 (29.6)				8 (29.6)	11 (40.7)
E	24	11 (45.8)	1 (4.2)		10 (41.7)		2 (8.3)
F	47	27 (57.5)			19 (40.4)		1 (2.1)

( ) percentage

Table 37

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather June 11, 1987

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High priority							
A	15	9 (60.0)		1 (6.7)	5 (33.3)		
B	24	16 (66.7)	2 (8.3)		4 (16.7)		2 (8.3)
C	22	14 (63.6)	1 (4.6)		3 (13.6)	3 (13.6)	1 (4.6)
Non-priority							
X	3	1 (33.3)		1 (33.3)	1 (33.3)		
Y	20	8 (40.0)	2 (10.0)	1 (5.0)	9 (45.0)		
Sanitary sewage							
D	12	8 (66.7)		1 (8.3)	1 (8.3)		2 (16.7)
E	29	5 (17.2)	3 (10.4)	2 (6.9)	16 (55.2)		3 (10.4)
F	0						

( ) percentage

Table 38

Percentage of *S. faecalis* Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Wet Weather Survey 1, July 14, 1987

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
Non-Priority							
X	18	8 (44.4)		1 (5.6)	8 (44.4)		1 (5.6)
Z	12	1 (8.3)	1 (8.3)	5 (41.7)	4 (33.3)		1 (8.3)
Run-offs							
P	10	2 (20.0)	2 (20.0)	2 (20.0)	3 (30.0)		1 (10.0)
Q	21	11 (52.4)	2 (9.5)	3 (14.3)	1 (4.8)		4 (19.0)

( ) percentage
P-storm water run-off Parliament & Carlton

Table 39

Percentage of *S. faecalis* Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Wet Weather Survey, September 18, 1987

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
X	2	2(100.0)					
G	10	3 (30.0)	2 (20.0)	3 (30.0)	2 (20.0)		

( ) percentage

Table 40

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Wet Weather Survey, Oct. 21, 1987

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
Non-Priority							
X	2		1 (50.0)	1 (50.0)			
Z	11	1 (9.1)	4 (36.4)	4 (36.4)	2 (18.2)		
Run-offs							
Q	3	1 (33.3)	1 (33.3)		1 (33.3)		
R	12	10 (83.3)	1 (8.3)	1 (8.3)			

( ) percentage

Table 41

Percentage of Serotypes of Pseudomonas aeruginosa from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey Oct. 21, 1986

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority storm sewer																				
A						3 (42.9)										3 (42.9)			1 (14.3)	7
B	1 (3.7)					23 (85.2)					1 (3.7)					1 (3.7)			1 (3.7)	27
C	23 (85.2)					2 (7.4)		2 (7.4)												27
% serotypes all samples	24 (39.3)					28 (45.9)		2 (3.3)			1 (1.6)					4 (6.6)			2 (3.3)	61
Sanitary sewage																				
D						7 (29.2)				17 (70.8)										24
E	1 (3.8)			1 (3.8)		4 (15.4)				2 (7.7)	17 (65.4)								1 (3.8)	26
F	2 (14.3)				2 (14.3)	8 (57.1)				1 (7.1)	1 (7.1)									14
% serotypes all samples	3 (4.7)			1 (1.6)	2 (3.2)	19 (29.7)				20 (31.3)	18 (28.1)								1 (1.6)	64

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 42

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Oct. 22, 1986

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority																				
A						20 (100.0)														20
B						20 (95.2)												1 (4.8)		21
C						2 (25.0)				1 (12.5)	4 (50.0)	1 (12.5)								8
% serotypes all samples						42 (85.7)				1 (2.0)	4 (8.2)	1 (2.0)						1 (2.0)		49
D	2 (11.1)									2 (11.1)								13 (72.2)	1 (5.6)	18
E	1 (4.2)					2 (8.4)				11 (45.8)								10 (41.7)		24
F		1 (4.5)		1 (4.5)		15 (68.2)	1 (4.5)											3 (13.6)	1 (4.5)	22
% serotypes all samples	3 (4.7)	1 (1.6)		1 (1.6)		17 (26.6)	1 (1.6)			13 (20.3)								26 (40.6)	2 (3.2)	64

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 43
Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey 4, Nov. 18, 1986

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority storm sewer																				
A						24 (100.0)														24
B						22 (100.0)														22
C						1 (100.0)														1
% serotypes all samples						47 (100.0)														47
Sanitary sewage																				
D	3 (12.5)					13 (54.2)				8 (33.3)										24
E	1 (4.5)					20 (91.0)												1 (4.5)		22
F						22 (100.0)														22
% serotypes all samples	4 (5.9)					55 (80.8)				8 (11.8)								1 (1.5)		68

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 44

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 10, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority																				
A	4 (16.0)			2 (8.0)	4 (16.0)	9 (36.0)			2 (8.0)							2 (8.0)		1 (4.0)	1 (4.0)	25
B	1 (3.3)					26 (86.7)												1 (3.3)	2 (6.7)	30
C	3 (10.7)	3 (10.7)		1 (3.6)		2 (7.1)			13 (46.4)	3 (10.7)									3 (10.7)	28
% serotypes all samples	8 (9.6)	3 (3.6)		3 (3.6)	4 (4.8)	37 (44.6)			15 (18.1)	3 (3.6)						2 (2.4)		2 (2.4)	6 (7.2)	83
Sanitary sewage																				
D	6 (20.7)				1 (3.4)	13 (44.8)					5 (17.2)					4 (13.8)				29
E	9 (39.1)		1 (4.3)			8 (34.8)				3 (13.0)						2 (8.7)				23
F	1 (3.6)					18 (64.3)				6 (21.4)	2 (7.1)								1 (3.6)	28
% serotypes all samples	16 (20.0)		1 (1.3)		1 (1.3)	39 (48.8)				9 (11.3)	7 (8.8)					6 (7.5)			1 (1.3)	80
Non-priority																				
X		1 (4.8)	1 (4.8)			7 (33.3)			5 (23.8)	3 (14.3)									4 (19.0)	21
Y		15 (50.0)	1 (3.3)	3 (10.0)		9 (30.0)		2 (6.7)												30
% serotypes all samples		16 (31.4)	2 (3.9)	3 (5.9)		16 (31.4)		2 (3.9)	5 (9.8)	3 (5.9)									4 (7.8)	51

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 45
Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 11, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority A						57 (96.6)												2 (3.4)		59
B		2 (3.3)				51 (85.0)												6 (10.0)	1 (1.7)	60
C		15 (25.0)		1 (1.7)		38 (63.3)			2 (3.3)									2 (3.3)	2 (3.3)	60
% serotypes all samples		17 (9.5)		1 (0.56)		146 (81.6)			2 (1.1)									10 (5.6)	3 (1.7)	179
Sanitary Sewage D	12 (20.0)		1 (1.7)	1 (1.7)	1 (1.7)	23 (38.3)				6 (10.0)						2 (3.3)		5 (8.3)	9 (15.0)	60
E	3 (5.0)	1 (1.7)	3 (5.0)	3 (5.0)	9 (15.0)	19 (31.7)			1 (1.7)	13 (21.7)	1 (1.7)					1 (1.7)		2 (3.3)	4 (6.6)	60
F	10 (16.9)		1 (1.7)	8 (13.6)	1 (1.7)	33 (55.9)			1 (1.7)	2 (3.4)								3 (5.1)		59
% serotypes all samples	25 (14.0)	1 (0.56)	5 (2.8)	12 (6.7)	11 (6.1)	75 (41.9)			2 (1.1)	21 (11.7)	1 (0.56)					3 (1.7)		10 (5.6)	13 (7.3)	179
Non-priority X	2 (22.2)					4 (44.4)			1 (11.1)									2 (22.2)		9
Y	2 (5.7)		1 (2.9)			5 (14.3)			4 (11.4)	21 (60.0)						1 (2.9)			1 (2.9)	35
% serotypes all samples	4 (9.1)		1 (2.3)			9 (20.5)			5 (11.4)	21 (47.7)						1 (2.3)		2 (4.5)	1 (2.3)	44

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 46
Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 12, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority																				
C	3 (11.1)		2 (7.4)			18 (66.7)												2 (7.4)	2 (7.4)	27
% serotypes all samples	3 (11.1)		2 (7.4)			18 (66.7)												2 (7.4)	2 (7.4)	27
Sanitary sewage																				
D	2 (7.7)	2 (7.7)	1 (3.8)			9 (34.6)				4 (15.4)				1 (3.8)				3 (11.5)	4 (15.4)	26
E	1 (3.3)					20 (66.6)				6 (20.0)								3 (10.0)		30
F	19 (70.0)	1 (3.7)	1 (3.7)			1 (3.7)				1 (3.7)	3 (11.1)								1 (3.7)	27
% serotypes all samples	22 (26.5)	3 (3.6)	2 (2.4)			30 (36.1)				11 (13.2)	3 (3.6)			1 (1.2)				6 (7.2)	5 (6.02)	83
Non-priority																				
X	3 (10.0)		5 (16.7)	1 (3.3)		16 (53.3)			3 (10.0)		1 (3.3)								1 (3.3)	30
Y	1 (3.4)			1 (3.4)		23 (79.3)												1 (3.4)	3 (10.3)	29
% serotypes all samples	4 (6.8)		5 (8.5)	2 (3.4)		39 (66.1)			3 (5.1)		1 (1.6)							1 (1.6)	4 (6.8)	59

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 47
Percentage of Serotypes of Pseudomonas aeruginosa from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey August 17, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority																				
A			1 (3.3)			4 (13.3)					24 (80.0)							1 (3.3)		30
B						21 (70.0)				2 (6.7)	1 (3.3)							3 (10.0)	3 (10.0)	30
C	1 (3.5)		3 (10.7)			13 (46.4)			5 (17.9)					1 (3.5)				5 (17.9)		28
% serotypes all samples	1 (1.1)		4 (4.4)			38 (43.2)			5 (5.6)	2 (2.3)	25 (28.4)			1 (1.1)				9 (10.2)	3 (3.4)	88
Sanitary sewage																				
D	3 (10.0)		1 (3.3)	1 (3.3)		6 (20.0)	1 (3.3)			9 (30.0)	1 (3.3)					1 (3.3)		3 (10.0)	4 (13.3)	30
E		1 (3.4)	6 (20.6)	2 (6.9)		14 (48.2)				1 (3.4)	1 (3.4)								4 (13.8)	29
F	10 (41.6)		6 (25.0)	2 (8.3)	1 (4.2)					1 (4.2)						1 (4.2)		1 (4.2)	2 (8.3)	24
% serotypes all samples	13 (15.6)	1 (1.2)	13 (15.6)	5 (6.02)	1 (1.2)	20 (24.0)	1 (1.2)			11 (13.3)	2 (2.4)					2 (2.4)		4 (4.8)	10 (12.0)	83
Non-priority																				
Y	8 (34.7)			1 (4.3)	2 (8.6)	2 (8.6)	1 (4.3)	1 (4.3)										7 (30.4)	1 (4.3)	23
% serotypes all samples	8 (34.7)			1 (4.3)	2 (8.6)	2 (8.6)	1 (4.3) (30.4)	1 (4.3) (4.3)										7	1	23

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 48

Percentage of Serotypes of Pseudomonas aeruginosa from High Priority Storm Sewers and Sanitary Sewage during Wet Weather Survey 1, July 14, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
Non-priority																				
X	46 (65.7)			7 (10)		15 (21.4)				1 (1.4)								1 (1.4)		70
Z	1 (2.8)	5 (13.8)	3 (8.33)		1 (2.8)	17 (47.2)		1 (2.8)		1 (2.8)		1 (2.8)						5 (13.8)	1 (2.8)	36
% serotypes all samples	47 (44.3)	5 (4.76)	3 (2.83)	7 (6.6)	1 (0.94)	32 (30.1)		1 (0.94)		2 (1.89)		1 (0.94)						6 (5.6)	1 (0.94)	106
Run-offs																				
P	8 (16.3)					39 (79.6)			1 (2.0)		1 (2.0)									49
Q	1 (5.26)					11 (57.9)				1 (5.26)								3 (15.8)	3 (15.8)	19
% serotypes all samples	9 (13.2)					50 (73.5)			1 (1.47)	1 (1.47)	1 (1.47)							3 (4.4)	3 (4.4)	68

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 49

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Wet Weather Survey Sept. 18, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
Non-priority																				
X	3 (2.8)	-	8 (7.5)	6 (5.6)	2 (1.9)	55 (51.4)	-	-	2 (1.9)	3 (2.8)	15 (14.0)	-	-	1 (.93)	-	1 (.93)	-	9 (8.4)	2 (1.9)	107
% serotypes all samples	3 (2.8)	-	8 (7.5)	6 (5.6)	2 (1.9)	55 (51.4)	-	-	2 (1.9)	3 (2.8)	15 (14.0)	-	-	1 (.93)	-	1 (.93)	-	9 (8.4)	2 (1.9)	107
Run-offs																				
G	17 (21.3)	-	2 (2.5)	1 (1.3)	-	39 (48.8)	-	2 (2.5)	2 (2.5)	5 (6.3)	-	-	-	-	-	1 (1.3)	-	3 (3.8)	8 (10.0)	80
% serotypes all samples	17 (21.3)	-	2 (2.5)	1 (1.3)	-	39 (48.8)	-	2 (2.5)	2 (2.5)	5 (6.3)	-	-	-	-	-	1 (1.3)	-	3 (3.8)	8 (10.0)	80

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 50

Percentage of Serotypes of _Pseudomonas aeruginosa_ from High Priority Storm Sewers and Sanitary Sewage during Wet Weather Survey Oct. 21, 1987

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
Non-priority																				
X	1 (7.1)		4 (28.6)			6 (42.9)					3 (21.4)									14
Z	2 (3.7)		3 (5.6)	2 (3.7)		22 (40.7)	2 (3.7)		13 (24.1)		3 (5.6)							3 (5.6)	4 (7.4)	54
% serotypes all samples	3 (4.4)		7 (10.3)	2 (2.9)		28 (41.2)	2 (2.9)		13 (19.1)		6 (8.8)							3 (4.4)	4 (5.9)	68
Q	3 (16.7)	1 (5.6)	1 (5.6)			7 (38.9)		1 (5.6)	1 (5.6)	1 (5.6)	1 (5.6)								2 (11.1)	18
R	3 (10.7)	1 (3.6)	3 (10.7)			15 (53.6)												4 (14.3)	2 (7.1)	28
% serotypes all samples	6 (13.0)	2 (4.3)	4 (8.7)			22 (47.8)		1 (2.2)	1 (2.2)	1 (2.2)	1 (2.2)							4 (8.7)	4 (8.7)	46

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 51

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA, AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM HIGH PRIORITY STORM SEWAGE DURING DRY WEATHER IN 1988

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	E. coli (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	Pseudomonas aeruginosa (m-PA)	Bifidobacterium (YN-17)	Clostridium perfringens (m-CP2)
June 20/88								
A	2.82×10^4	1.10×10^4	4.27×10^3	1.15×10^3	6.6	5.01×10^2		NA
B	2.00×10^3	1.82×10^3	1.70×10^3	5.37×10^2	1.18	6.46×10^1		NA
C	1.70×10^3	1.51×10^3	7.59×10^2	3.98×10^2	2.24	1.00×10^1		NA
June 21/88								
A	3.24×10^3	8.91×10^2	1.48×10^3	9.3×10^3	2.19	2.40×10^1		NA
B	9.77×10^3	5.62×10^3	1.70×10^4	8.13×10^3	0.57	3.16×10^2		NA
C	9.55×10^3	4.68×10^3	1.48×10^5	1.15×10^5	0.06	2.75×10^2		NA
August 15/88								
A	1.02×10^5	7.94×10^3	6.17×10^4	6.60×10^4	1.65	1.35×10^3		NA
C	2.88×10^8	1.00×10^7	4.68×10^4	2.04×10^4	6153.8	7.41		NA
August 22/88								
A	1.41×10^5	2.51×10^4	2.75×10^4	1.55×10^4	5.13	9.33×10^2		NA
B	1.00×10^4	5.25×10^3	2.00×10^3	2.57×10^2	5.00	0.0		NA
C	6.46×10^4	1.58×10^4	9.55×10^4	5.62×10^4	0.68	0.0	1.26×10^5	NA

NA - Not analyzed. Volume shortage of sample.

Table 52

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM SANITARY SEWAGE DURING DRY WEATHER SURVEY IN 1988

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	*E. coli* (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	*Pseudomonas aeruginosa* (m-PA)	Bifidobacterium (YN-17)	*Clostridium perfringens* (m-CP2)
June 20								
D	9.77×10^5	6.91×10^5	1.74×10^5	1.45×10^5	5.61	2.69×10^4		
E	4.47×10^6	3.80×10^6	8.51×10^2	1.23×10^3	5252.6	7.08×10^3		
F	3.31×10^4	3.31×10^4	1.51×10^4	6.46×10^3	2.19	1.00×10^4	1.58×10^4	
June 28								
E			7.94×10^3	1.62×10^3				
F						6.31		
August 15								
D	2.29×10^8	2.00×10^8	3.09×10^6	6.76×10^4	74.1	1.32×10^5		
E			2.95×10^4	1.20×10^4		2.82×10^4		
F	1.58×10^4	8.13×10^3	2.82×10^3	1.91×10^3	5.6	8.71×10^1		
August 22								
D	7.76×10^4	7.08×10^7	1.12×10^6	1.23×10^6	0.07	6.61×10^4		
E	1.05×10^7	6.31×10^6	1.58×10^4	1.70×10^3	664.6			
F	1.26×10^4	1.10×10^4	2.04×10^3	1.62×10^3	6.18	2.57×10^1		

NA - not analyzed. Volume shortage of sample.

Table 53

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, ESCHERICHIA COLI, FECAL STREPTOCOCCI, ENTEROCOCCI, PSEUDOMONAS AERUGINOSA, BIFIDOBACTERIA, AND CLOSTRIDIUM PERFRINGENS RECOVERED FROM NON-PRIORITY STORM SEWAGE DURING DRY WEATHER IN 1988

Parameter per 100 mL of Sample

DATE/SAMPLE	Fecal coliforms (m-Tec)	*E. coli* (urease)	Fecal streptococci (m-Ent)	Enterococci (m-E)	FC:FS ratio	*Pseudomonas aeruginosa* (m-PA)	Bifidobacterium (YN-17)	*Clostridium perfringens* (m-CP2)
June 20								
Y	7.76×10^2	5.89×10^2	1.70×10^3	4.47×10^2	0.46	3.80×10^1		NA
June 21								
Y	7.76×10^2	5.25×10^2	3.39×10^3	1.58×10^3	0.23	1.91×10^1		NA
June 28								
X	9.77×10^2	7.94×10^2	4.57×10^4	3.16×10^4	0.02	1.78×10^1		NA
Y						3.63×10^1		NA
Z	2.29×10^2	1.48×10^2	8.13×10^3	2.75×10^2	0.03	1.29×10^1	7.59×10^3	NA
August 15								
X			5.01×10^3	1.23×10^2	-	9.3×10^2		NA
Y	8.91×10^4	2.81×10^4	1.05×10^5	5.25×10^4	0.85	2.51×10^4		NA
August 22								
X	4.07×10^5	3.63×10^4	1.58×10^3	6.61×10^2	257.6	4.47×10^3		NA
Y	1.91×10^4	1.45×10^4	4.57×10^2	NA	41.8	1.41×10^2	2.29×10^4	NA
Z	2.88×10^4	1.02×10^4	3.24×10^2	2.34×10^2	88.9	3.80×10^2		NA

NA - Not analyzed. Volume shortage of sample.

Table 54

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 20, 1988

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NS	Rough	Total
High priority storm sewer																				
A						3 (75.0)													1 (25.0)	4
B						5 (100.0)														5
C						3 (75.0)					1 (25.0)									4
% serotypes all samples						11 (84.6)					1 (7.7)								1 (7.7)	13
Sanitary sewage																				
D								1 (25.0)		3 (75.0)										4
E						2 (40.0)				3 (60.0)										5
% serotypes all samples						2 (22.2)		1 (11.1)		6 (66.7)										9
Non-Priority																				
Y	2 (50.0)		1 (25.0)								1 (25.0)									4
% serotypes all samples	2 (50.0)		1 (25.0)								1 (25.0)									4

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 55

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 21, 1988

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority storm sewer																				
A	3 (100.0)																			3
B						4 (100.0)														4
C	2 (33.3)					2 (33.3)			1 (16.7)	1 (16.7)										6
% serotypes all samples	5 (38.5)					6 (46.2)			1 (7.7)	1 (7.7)										13
Sanitary sewage																				
D										2 (66.7)	1 (33.3)									3
E	1 (33.3)																		2 (66.7)	3
% serotypes all samples	1 (16.7)									2 (33.3)	1 (16.7)								2 (33.3)	6
Non-Priority																				
Y			1 (100.0)																	1
% serotypes all samples			1 (100.0)																	1

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 56

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey June 28, 1988

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
Sanitary sewage																				
F						1 (100.0)														1
% serotypes all samples						1 (100.0)														1
Non-Priority Stormsewers X						2 (40.0)				1 (20.0)									2 (40.0)	5
Y	2 (100.0)																			2
Z						3 (50.0)			3 (50.0)											6
% serotypes all samples																				

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 57

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey August 15, 1988

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority storm sewer																				
A						4 (100.0)														4
C			1 (50.0)						1 (50.0)											2
% serotypes all samples			1 (16.7)			4 (66.7)			1 (16.7)											6
Sanitary sewage D			1 (33.3)			1 (33.3)													1 (33.3)	3
E						4 (100.0)														4
F						2 (66.6)													1 (33.3)	3
% serotypes all samples			1 (10.0)			7 (70.0)													2 (20.0)	10
Non-Priority Storm sewers X	1 (25.0)					2 (50.0)			1 (25.0)											4
Y						1 (100.0)														1
% serotypes all samples	1 (20.0)					3 (60.0)			1 (20.0)											5

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 58

Percentage of Serotypes of *Pseudomonas aeruginosa* from High Priority Storm Sewers and Sanitary Sewage during Dry Weather Survey August 22, 1988

Sewage Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	NT	Rough	Total
High priority Storm sewer A						6 (100.0)														6
% serotypes all samples						6 (100.0)														6
Sanitary sewage D				1 (20.0)		4 (80.0)														5
F						1 (33.3)													2 (66.6)	3
% serotypes all samples				1 (12.5)		5 (62.5)													2 (25.0)	8
Non-Priority X						3 (100.0)														3
Y	2 (40.0)		1 (20.0)			1 (20.0)					1 (20.0)									5
Z	1 (33.3)					1 (33.3)				1 (33.3)										3
% serotypes all samples	3 (27.3)		1 (9.1)			5 (45.5)				1 (9.1)	1 (9.1)									11

( ) percentage
NT - nontypable
Rough - more than 1 serotype

Table 59

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather June 20, 1988

Species and variant	High Priority			Non-Priority	Sanitary Sewage		
	A [17]	B [10]	C [11]	Y [22]	D [3]	E [5]	F [10]
S. faecalis var.							
faecalis			1 (9.1)	1 (4.5)	1 (33.3)		-
liquefaciens			-	5 (22.7)	-		1 (10.0)
zymogenes	1 (5.9)		6 (54.5)	-	2 (66.6)	1 (20.0)	4 (40.0)
S. faecium	12 (70.6)	3 (30.0)	4 (36.4)	8 (36.4)		1 (20.0)	3 (30.0)
S. faecium var. casseliflavus	4 (23.5)	7 (70.0)		5 (22.7)		3 (60.0)	2 (20.0)
S. durans				2 (9.1)			
S. avium							
S. bovis							
not FS				1 (4.5)			
FS							
S. lactis							
S. equinus							
S. galactis							

( ) percentage of total
[ ] number of isolates identified

Table 60

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather June 21, 1988

Species and variant	High Priority			Non-Priority
	A [14]	B [14]	C [7]	Y [45]
S. faecalis var.				
faecalis				4 (8.9)
liquefaciens				5 (11.1)
zymogenes				1 (2.2)
S. faecium	1 (7.1)	14(100.0)	4 (57.1)	22 (48.9)
S. faecium var. casseliflavus	11 (78.6)		1 (14.3)	12 (26.7)
S. durans	2 (14.2)		2 (28.6)	1 (2.2)
S. avium				
S. bovis				
not FS				
FS				
S. lactis				
S. equinus				
S. galactis				

( ) percentage of total
[ ] number of isolates identified

Table 61

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather June 28, 1988

Species and variant	Non-Priority		Sanitary Sewage
	X [31]	Z [28]	E [5]
S. faecalis var.			
faecalis	2 (6.5)	9 (32.1)	1 (20.0)
liquefaciens	3 (9.7)	2 (7.1)	-
zymogenes	1 (3.2)	1 (3.6)	-
S. faecium	4 (12.9)	11 (39.3)	4 (80.0)
S. faecium var.			
casseliflavus	9 (29.0)	5 (17.9)	
S. durans	12 (38.7)		
S. avium			
S. bovis			
not FS			
FS			
S. lactis			
S. equinus			
S. galactis			

( ) percentage of total
[ ] number of isolates identified

Table 62

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather August 15, 1988

Species and variant	High Priority			Non-Priority		Sanitary Sewage		
	A [12]	B	C [9]	X [27]	Y [10]	D [14]	E [7]	F [9]
S. faecalis var.								
faecalis			1 (11.1)	1 (3.7)	1 (10.0)	-	1 (14.3)	-
liquefaciens			1 (11.1)	1 (3.7)	2 (20.0)	3 (21.4)	-	-
zymogenes			4 (44.4)	-	-	2 (14.3)	2 (28.6)	6 (66.7)
S. faecium	9 (75.0)		2 (22.2)	3 (11.1)	1 (10.0)	2 (14.3)	3 (42.9)	2 (22.2)
S. faecium var.								
casseliflavus	3 (25.0)		1 (11.1)	17 (63.0)	3 (30.0)	7 (50.0)	1 (14.3)	1 (11.1)
S. durans				2 (7.4)				
S. avium								
S. bovis								
not FS								
FS								
S. lactis				3 (11.1)				
S. equinus								
S. galactis								

( ) percentage of total
[ ] number of isolates identified

Table 63

Fecal Streptococci Populations Recovered from Storm Sewers and Sanitary Sewage during Dry Weather August 22, 1988

Species and variant	High Priority		Non-Priority		
	A [6]	C [6]	X [13]	Y [10]	Z [28]
S. faecalis var.					
faecalis		-	-	3 (30.0)	-
liquefaciens		-	-	1 (10.0)	5 (17.9)
zymogenes		2 (33.3)	-	-	1 (3.6)
S. faecium	6(100.0)	2 (33.3)	2 (15.4)		1 (3.6)
S. faecium var.					
casseliflavus		2 (33.3)	7 (53.9)	1 (10.0)	19 (67.9)
S. durans			4 (30.8)	5 (50.0)	2 (7.1)
S. avium					
S. bovis					
not FS					
FS					
S. lactis					
S. equinus					
S. galactis					

( ) percentage of total
[ ] number of isolates identified

Table 64

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather June 20, 1988

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High Priority							
A	1			1(100.0)			
B	0						
C	7	5 (71.4)	1 (14.3)	1 (14.3)			
Non-Priority							
Y	6	1 (16.7)		1 (16.7)	4 (66.7)		
Sanitary Sewage							
D	3	2 (66.6)			1 (33.3)		
E	1			1(100.0)			
F	5	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)		

( ) percentage

Table 65

Percentage of _S. faecalis_ Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather June 21, 1988

Sample Location		Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High Priority								
	A	0						
	B	0						
	C	0						
Non-Priority								
	Y	10	5 (50.0)		1 (10.0)	2 (20.0)	2 (20.0)	

( ) percentage

Table 66

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather June 28, 1988

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
Non-Priority							
X	6	2 (33.3)	2 (33.3)	1 (16.7)	1 (16.7)		
Z	12		12(100.00)				
Sanitary Sewage							
E	1	1(100.0)					

( ) percentage

Table 67

Percentage of S. faecalis Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather August 15, 1988

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein- ization	Digestion	No Reaction
High Priority							
A	0						
C	6	4 (66.7)	1 (16.7)			1 (16.7)	
Non-Priority							
X	2		1 (50.0)	1 (50.0)			
Y	3	2 (66.7)		1 (33.3)			
Sanitary Sewage							
D	5	1 (20.0)		1 (20.0)		3 (60.0)	
E	3		2 (66.7)		1 (33.3)		
F	6			5 (83.3)	1 (16.7)		

( ) percentage

Table 68

Percentage of *S. faecalis* Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk from Dry Weather August 22, 1988

Sample Location	Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High Priority							
A	0						
B	0						
C	2	1 (50.0)			1 (50.0)		
Non-Priority							
X	0						
Y	4		4 (100.0)				
Z	6	2 (33.3)		1 (16.7)	3 (50.0)		

( ) percentage

Table 69

Summary of the Percentage of _S. faecalis_ Isolates Producing Acid Curds or Proteinization Reactions in Litmus Milk in 1988 Dry Weather Samples

Sample Location		Total Isolates	Acid Curd	Reduction	Alkaline Curd	Protein-ization	Digestion	No Reaction
High Priority Storm Sewage								
	A	1			1(100.0)			
	B	0						
	C	15	10 (66.7)	2 (13.3)	1 (6.7)	1 (6.7)	1 (6.7)	
Non-Priority Storm Sewage								
	X	8	2 (25.0)	3 (37.5)	2 (25.0)	1 (12.5)		
	Y	23	8 (34.8)	4 (17.4)	3 (13.0)	6 (26.1)	2 (8.7)	
	Z	18	2 (11.1)	12 (66.7)	1 (5.5)	3 (16.7)		
Sanitary Sewage								
	D	8	3 (37.5)		1 (12.5)	1 (12.5)	3 (37.5)	
	E	5	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)		
	F	11	1 (9.1)	2 (18.2)	6 (54.5)	2 (18.2)		

( ) percentage

APPENDIX B

COMPARATIVE STUDY OF THE SURVIVAL OF INDICATOR BACTERIAL SPECIES

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LIST OF ABBREVIATIONS

Organisms:

E. coli - *Escherichia coli*

B. breve - *Bifidobacterium breve*

B. longum - *Bifidobacterium longum*

P. aeruginosa - *Pseudomonas aeruginosa*

S. faecalis var. *faecalis* - *Streptococcus faecalis* variety *faecalis*

S. faecium var. *casseliflavus* - *Streptococcus faecium* variety *casseliflavus*

CFU - Colony forming unit

^{o}C - Degrees celsius

dH$_2$O - Distilled water

E - Exponential

pH - Hydrogen ion concentration

mL - millilitre

ppm - Parts per million

PSI - Pounds per square inch

vs. - versus

M - molar

INTRODUCTION

Fundamental to the interpretation of data concerning surface water quality is an understanding of the growth cycles and viability of fecal indicator bacteria and enteric pathogens disseminated by waterways. In the recent past public awareness pertaining to the contamination of surface waters has resulted in increasing efforts to control this type of pollution. The potential health hazard, in which waterways may act as both a vehicle and a reservoir for agents of infectious disease, is apparent. The prevention of epidemics caused by such organisms as Salmonella typhi, Salmonella typhimurium, Vibrio cholerae, Shigella species, Giardia lamblia, enterotoxigenic E. coli and P. aeruginosa may only take place with the adequate control of fecal contamination (Gyles, 1984).

The main concern of this study deals with the comparative analysis of the life cycle of E. coli, P. aeruginosa, S. faecalis var. faecalis, S. faecium and B. longum. The theoretical aspect of this investigation is concerned with obtaining an organism of short life duration so that it may be used as an indicator of recent fecal contamination. Although the detection of fresh fecal contaminants within any surface water body is paramount, the impetus for this research came from the ever growing problem of storm water sewage contamination. It is for this reason that growth curves for the indicator organisms were established at both room temperature and 15^{o}C (approximate sewer temperature).

The original design strategy behind storm water sewage channels was the alleviation of excessive runoff - water from urbanized areas. Theoretically these storm sewers should have no human fecal input, however, as reported by the Toronto Area Watershed Management (TAWM), this is not the case. Fecal pollution within the Humber and Don Rivers does result in storm water sewage serving as a contributor.

The organisms selected for this study were chosen for the following reasons:

E. coli

- This organism is constantly found in the human intestine in large numbers.
- The fate of the coliform bacteria reasonably reflects that of the pathogenic bacteria, although the life expectancy of the fecal coliforms is normally longer than that of intestinal pathogens.
- This organism is easy to isolate and enumerate in the laboratory environment and is normally not pathogenic (Schuettpelz, 1969).

S. faecalis var. faecalis / S. faecium

- A high percentage of the strains have properties which enable their source to be identified with considerable certainty.
- Members of the fecal streptococci group are often used to signify the presence of intestinal pathogens (McFeters et al., 1974).

P. aeruginosa

- Ringen and Drake (1952) have shown that this organism was not isolated from locations free of human habitation or waste material.
- This opportunistic pathogen is particularly associated with the high incidence of otitis externa in surface water related areas (Levin and Cabelli, 1972).

B. longum

- The strictly anaerobic requirement of this organism makes its inclusion in a study of growth within a sewer environment of interest.
- A relatively small amount of work has been done on this organism and its membership in the group of fecal organisms.

The method of choice for the enumeration of the indicator organisms was that of membrane filtration. This technique was selected because of the relative simplicity of the procedure, the capability of obtaining results within 24 hours, instead of the 48 to 96 hours demanded by the Most Probable Number test and finally, the larger volume of samples which may be analyzed making the obtained results more representative.

The second portion of this survival research project concerns iself with the effect of chlorination on the following organisms: E. coli, P. aeruginosa, S. faecium var. casseliflavus and B. breve. The lethality of uncombined chlorine, in the form of unionized hypochlorous acid has made this chemical the most widely used reagent for the disinfection of water distribution systems and reservoirs (Ridgway and Olson, 1982). Of more recent interest, however, has been the possible application of the method of chlorination for purposes of sterilization. Factors such as concentration of the chlorine used, the pH of the environment in which the chlorine is dispensed and the free chlorine residual must all be considered when analysing the effectiveness of this agent as a bactericide (Seyfried and Fraser, 1979).

The action of chlorine, from the chemical point of view, may be summarized as follows:

1. Hydrolysis of chlorine:

   $Cl_2 + HOH = HOCl + H^+ + Cl^-$

   Above pH values of 5.0, the hypochlorous acid (HOCl) dissociates in aqueous solution and forms an equilibrium with hydrogen and hypochlorite ions, with the relative amount of each species dependent upon the pH.

2. Dissociation of hypochlorous acid:

   $HOCl = H^+ + OCl^-$

   The bactericidal activity is a result of the hypochlorous acid which is free chlorine, while the hypochlorite ion has limited killing ability, but acts as a reservoir of available chlorine.

3. Combined or "stabilized" chlorine:

This is the reaction of chlorine with such materials as ammonia or its salts, cyanuric acid, sulfamic acid or urea. Chlorine in this form is virtually ineffective, having a killing time of approximately one thirtieth that of hypochlorous acid (Black et al., 1970).

The final point of interest concerning the action of chlorine is related to its biochemical action upon the bacterial cell. It is at this point that differences in lethality exist, when environmental conditions are kept constant between organisms. Recent proposals, by Camper and McFeters (1979) as to the mode of action of chlorine on the bacterial cell include:

- unbalanced metabolism after the destruction of key enzymes
- disruption of protein synthesis
- oxidative decarboxylation of amino acids
- reactions with nucleic acids, purines and pyrimidines
- formation of chloro derivatives of cytosine
- creation of chromosomal abberations
- induction of deoxyribonucleic acid lesions with the accompanying loss of deoxyribonucleic acid transforming ability
- inhibition of oxygen uptake and oxidative phosphorylation coupled with the leakage of some macromolecules

OBJECTIVE OF RESEARCH

The objectives of this research project were to:

1. Study the growth cycle of *E. coli*, *P. aeruginosa*, *S. faecalis* var. *faecalis*, *S. faecium* and *B. longum*. The results of this comparative analysis may then be applied to the development of a methodology for detecting the source of sanitary waste pollution in surface water bodies, since the presence of a short life expectancy organism will be indicative of recent pollution.

2. Of growing interest is the use of chlorine, not only as a disinfectant, but also as a potential sterilant. Manipulation of the concentration of chlorine, as well as the hydrogen ion concentration of the environmental setting, will provide insight into the effective lethality of chlorine on *E. coli*, *P. aeruginosa*, *S. faecium* var. *casseliflavus*, and *B. breve*.

MATERIALS AND METHODS

3.1 Bacterial cultures:

Seven bacterial cultures were used in this study.

- Four laboratory strains which included *E. coli*, *P. aeruginosa*, *S. faecalis* var. *faecalis*, and *S. faecium*.
- Two environmental isolates which included *B. longum* and *S. faecium* var. *casseliflavus*.
- One fecal sample isolate, *B. breve*, taken from a chicken.

The environmental isolates and the fecal sample isolate were positively identified using biochemical testing procedures, as outlined in Bergey's Manual of Determinative Microbiology (1985).

3.2 Growth cycle determination:

- *E. coli*, *P. aeruginosa*, *S. faecalis* var. *faecalis* and *S. faecium* were all grown for 24 hrs., aerobically, in 10mL of nutrient broth, at 35°C.
- *B. longum* was grown for 48 hrs., anaerobically, in 10mL of MRS broth at 35°C.
- Two 250mL culture flasks were autoclaved for 15 minutes at 121°C / 15 PSI. To these flasks, 90mL of filter sterilized pond water was then aseptically added.
- To ensure that sterility was achieved, spread plates of the sterilized water were made on nutrient agar.
- Once these culture flasks were prepared, the five cultures

Table B-1

Media and Incubation Parameters for Enumeration of Selected Bacterial Groups

BACTERIAL GROUP	MEDIUM*	INCUBAT. TEMP °C	TIME (HRS)	TARGET COLONY MORPHOLOGY
E. Coli	m-TEC	44.5	23 + 1	- flat greenish/yellow colonies - circular
	NA	35	24	- small beige colonies - circular
P. aeruginosa	m-PA	41.5	48	- convex brownish-green or tan - circular
	NA	35	24	- spread out and of irregular form
S. faecalis var. faecalis	m-ENT	35	48	- maroon, red, or pink colonies - circular and raised
S. faecium	m-E	41.5	48	- blue/pink colonies - pulvinate to umbinate
S. faecium var. casseliflavus	BHI	35	24	- very small yellow colonies - convex to pulvinate
B. longum	YN17 Blue	35	48	- smooth to undulating surface - convex to pulvinate - soft, moist, slimy blue colonies
B. breve	MRS	35	24	- convex to pulvinate - ivory coloured - muccoid and soft

were each diluted in 10mL of their respective broths and then aseptically added to the culture flasks.

- These dilutions were prepared so as to start with an initial colony forming units per mL of approximately 10E5 to 10E6.
- The bacterial culture dilultions were made in duplicate, as were the culture flasks, in order that the growth cycles could be examined at both 15°C and room temperature.
- Both the temperature of the 15°C incubator, as well as the temperature of the laboratory were recorded daily:

  Room temperature range - 23°C +/- 2°C

  15°C incubator - 15°C +/- 2°C, with the exception of Day 9 of the study when the incubator temperature fell to 8°C.

3.3 Membrane filtration technique for the isolation of the test organisms.

- The procedure followed for the membrane filtration was that as outlined in (Standard Methods for the Examination of Water and Waste Water, 1971).
- Samples were removed from both the 15°C and the room temperature culture flasks over a 40 day experimental period.
- The first determination (Day 0) was taken immediately following the initial incubation of the bacterial cultures within the pond water.
- Sample filtration, for the purpose of enumeration, was always carried out between 9:00AM and 12:00PM and the determination dates were recorded.
- Samples were first diluted in 99mL phosphate buffer dilution blanks (see Appendix) and then filtered (Gelman filters).
- Approximately 100mL of phosphate buffer wash water (see Appendix) were

divided into three aliquots for consecutive rinsing of the sample and membrane filtration apparatus.

- After washing the membrane filters were placed on the growth medium, taking care to ensure that no air bubbles were trapped under the filter.
- The growth medium, incubation parameters and target morphology for all of the test organisms may be found in Table 1.

3.4 Enumeration of colony forming units per mL of test bacterial cultures:

- For all organisms, with the exception of *B. longum*, target colony morphology was specific to the isolating growth medium.
- The ability of fecal streptococci to grow on the YN17 Blue (*Bifidobacterium* isolating) medium made morphological examination of typical colonies from this medium essential.
- Typical colonies were selected from the membrane filter, from the YN17 Blue medium and microscope slide preparations were made.
- *Bifidobacterium* characteristic morphology; long curved, club-shaped, swollen or dumb-bell shaped rods, which may also be bifurcated.
- At the point in time when this type of cell morphology was no longer seen, typical colonies were selected and exposed to biochemical testing (Bergey's Manual of Determinative Microbiology, 1985).
- The two fecal streptococci, *S. faecalis* var. *faecalis* and *S. faecium*, were both included into the study so that it could be determined which had a shorter life span. Typical colonies were selected from the m-E medium (see Appendix) and biochemically tested so as to permit the calculation of a ratio of surviving numbers of the two fecal streptococci.
- The fecal streptococci were also grown on both m-Ent and m-E (see

Appendix) so as to compare the consistency of counts between the colony forming units on the two different media.

- The appearance of the colonies on m-E agar was variable, and biochemical tests were performed on two different types of colonies in order to determine whether these differences represented the two different fecal streptococci.

3.5 Chlorine test procedure:

- The bacterial effect of chlorine on the test organisms was determined using the experimental procedure of Seyfried and Fraser (1980).
- All glassware which came into contact with the experimental chlorine concentrations was acid-washed and then treated overnight with a calcium hypochlorite solution of concentration 0.01g per 1000mL.
- A stock chlorine solution of 0.5g calcium hypochlorite to 500mL of dH_2O was prepared. A sterile sodium thiosulfate solution (0.35g / 500mL) was also prepared and was used to neutralize the chlorine.
- Reaction tubes were filled with 8mL of dH_2O and then autoclaved for 15 minutes at 121°C / 15 PSI. With sterilized 1M sodium hydroxide and 1M hydrochloric acid the pH of the reaction vessels was then adjusted to [illegible] and 6.0, respectively.
- E. coli. P. aeruginosa and S. faecium var. casseliflavus were all grown for 24 hours, aerobically, in 10mL of nutrient broth at 35°C.
- B. breve was grown anaerobically for 24 hours in 10mL of MRS broth at 35°C.
- Two variables were incorporated into the experimentation of the

bactericidal effect of chlorine on the test organisms, through the following experimental trials:

(A) Effect of chlorine at a concentration of 0.2 ppm and a pH of 6.0

(B) Effect of chlorine at a concentration of 0.2 ppm and a pH of 9.0

(C) Effect of chlorine at a concentration of 0.4 ppm and a pH of 6.0

(D) Effect of chlorine at a concentration of 0.4 ppm and a pH of 9.0

- Bacterial cultures were centrifuged and then washed twice with 5mL aliquots of sterile dH_2O. The washed cultures were then resuspended in 5mL of sterile dH_2O and thoroughly mixed.
- The estimation of the chlorine concentration was done before each experimental trial and made use of the diethyl-p-phenylenediamine (DPD) test (American Public Health Association, 1971).
- At this point 1.0mL of culture was removed and diluted in 99mL of phosphate buffer dilution blank. This suspension was then placed on ice for later further dilution and subsequent membrane filtration. In this manner the initial concentration of bacteria per mL could be calculated.
- The test system consisted of 8mL of sterile pH adjusted dH_2O, 1mL of test culture suspension and 1mL of chlorine.
- The cells were exposed to the particular chlorine concentration for 1 minute. After this contact period 1mL of the neutralizing solution of 1M sodium thiosulfate was added to stop the reaction.
- 1mL of suspension was then removed from the reaction tube and placed in the 99mL phosphate buffer dilution blanks.
- Membrane filtration was performed first on the chlorine exposed cells and then on the initial cells which were on ice until this point. Each test

organism was filtered individually, at the "before and after" exposures, so as to reduce the possibility of any cross contamination.

- Referring to Table 1, the growth media, incubation parameters and typical colony morphology may be seen (Note: In this case selective media was not used. Instead, less stressful media was chosen; nutrient agar, BHI agar and MRS agar).
- The experimental procedure was performed at a temperature of 23 +/- $2^{o}C$.

RESULTS

4.1 Growth cycle determination:

Tables 2 and 3 show the growth of *E. coli*, *P. aeruginosa*, *S. faecalis* var. *faecalis* / *S. faecium* on m-Ent and m-E media, and *B. longum*. Enumeration of test bacterial cultures was performed at room temperature and 15°C (Table 2 and Table 3, respectively).

As can be seen in the data of the room temperature determination, the fecal streptococci attained the highest numbers of colony forming units per mL. Although all organisms showed a period of substantial increase in growth, this period was extremely short for *B. longum*, lasting for only the first day. In conjunction with this, *B. longum* was the first organism to die off; as well, *P. aeruginosa* was the only other organism to die before the end of the 40 day experimentation period. Some fluctuation in growth was recorded, namely the data collected does not reflect a steady increase in growth followed by a decline period. Examination of the results of the fecal streptococci illustrate a high degree of consistency between the colony forming unit per mL values on m-Ent with that of m-E.

At the 15°C determination, the most striking feature is the rapid "die-off" of *E. coli*, *P. aeruginosa* and *B. longum*. These three organisms all perished sometime between Day 8 and Day 13. As with the room temperature determination, the fecal streptococci again were most abundant; however, the numbers obtained at 15°C were not quite as high as those at room temperature. Another notable occurrence is that,

Table B-2

Colony Forming Units per Millilitre at Room Temperature

TIME (days)	*E. coli*	*P. aeruginosa*	*Strep.* on Ment	*Strep.* on ME	*B. longum*
0	1.800E+05	2.695E+05	1.320E+06	1.815E+06	5.840E+05
1	2.330E+07	1.090E+06	3.980E+06	3.730E+06	7.610E+05
4	3.750E+07	1.875E+06	4.390E+07	5.540E+07	5.370E+05
5	3.070E+07	3.480E+06	1.223E+08	1.383E+08	5.025E+05
6	1.925E+07	1.800E+06	1.335E+08	1.250E+08	1.395E+05
7	4.900E+07	1.123E+06	6.333E+07	1.405E+08	2.230E+05
11	4.120E+07	2.880E+07	5.750E+07	1.363E+08	9.720E+04
13	3.950E+07	4.000E+07	2.330E+09	6.150E+07	0.000E+00
15	4.500E+07	7.850E+08	3.790E+08	1.920E+09	0.000E+00
19	2.100E+07	8.640E+08	3.180E+08	5.960E+08	0.000E+00
21	1.840E+07	3.500E+07	2.880E+07	1.660E+08	0.000E+00
25	1.760E+07	4.740E+06	2.610E+07	3.910E+07	0.000E+00
27	1.400E+07	6.320E+05	2.800E+07	4.500E+07	0.000E+00
29	9.200E+06	4.640E+04	2.700E+07	3.900E+07	0.000E+00
36	6.800E+06	1.300E+02	2.400E+07	3.000E+07	0.000E+00
40	8.340E+05	0.000E+00	1.240E+04	1.840E+07	0.000E+00

Table B-3

Colony Forming Units per Millilitre at 15 degrees Celsius

TIME (days)	*E. coli*	*P. aeruginosa*	*Strep.* on Ment	*Strep.* on ME	*B. longum*
0	2.020E+05	3.170E+05	1.350E+06	1.625E+06	4.900E+05
1	1.420E+05	1.903E+05	1.453E+06	1.500E+06	4.450E+05
4	1.260E+05	2.760E+05	4.640E+06	4.685E+06	4.210E+05
5	8.850E+04	1.085E+05	9.850E+06	1.005E+07	1.873E+05
6	2.020E+05	3.245E+05	4.800E+07	3.070E+07	9.730E+04
7	2.087E+06	3.200E+04	3.270E+08	2.880E+08	8.060E+04
11	4.100E+04	0.000E+00	1.900E+08	2.437E+08	2.120E+04
13	2.000E+02	0.000E+00	2.720E+08	2.725E+08	0.000E+00
15	0.000E+00	0.000E+00	2.310E+08	2.040E+08	0.000E+00
19	0.000E+00	0.000E+00	1.130E+08	1.090E+07	0.000E+00
21	0.000E+00	0.000E+00	7.650E+06	9.130E+06	0.000E+00
25	0.000E+00	0.000E+00	1.190E+04	1.850E+04	0.000E+00
27	0.000E+00	0.000E+00	2.400E+04	2.200E+04	0.000E+00
29	0.000E+00	0.000E+00	1.900E+04	2.500E+04	0.000E+00
36	0.000E+00	0.000E+00	1.400E+04	2.800E+04	0.000E+00
40	0.000E+00	0.000E+00	1.240E+04	2.330E+04	0.000E+00

with the exception of the fecal streptococci on m-Ent, all other cases showed a decline immediately after incubation was initiated. Again, a degree of fluctuation within the growth cycle of the organisms was observed upon enumeration.

In the case of B. longum, at both room temperature and 15°C, microscopic slide preparations of colonies from the Day 11 determination did possess cells of characteristic Bifidobacterium morphology. On the Day 13 enumeration, however, morphological analysis proved negative for Bifidobacterium and for this reason four typical colonies were selected from a YN17 plate containing 5 colonies, for biochemical testing (Bergey's Manual of Determinative Microbiology, 1985). The results obtained were all negative for Bifidobacterium, but positive for Streptococcus. Due to the absence of positively identifiable Bifidobacterium all colonies which did grow were not enumerated and it was assumed that B. longum no longer existed.

Figures 1 through 10 represent the graphical analysis of the results recorded in Tables 1 and 2. The following is an explanation of what the respective Figures depict:

Figures 1 and 2 are comparative graphs of the test organisms at both room temperature and 15°C. (Note: These Figures, as with all others in which the fecal streptococci are found, do not distinguish between S. faecalis var. faecalis and S. faecium but instead compare the combined fecal streptococci as to their growth on either m-Ent or m-E growth media). As can be seen on these graphs, the values for the fecal streptococci are enormous in comparison to the other organisms. For this reason, magnifications of these graphs were required and may be found in Figures 8 and 9. The fluctuations in growth are more readily apparent when viewing the graphs, but in an attempt to compensate for those days on which determinations were not taken, the "best-fit" line was drawn through the points. Two final points worthy of mention include the rapid "die-off" of all organisms but the fecal streptococci, at 15°C. Secondly, the fact that only *E. coli* demonstrates a period of growth followed by a short period of "die-off" and then another resurgence, at both temperature settings.

Figures 3 through 7 plot the results obtained at room temperature against those of 15°C, for each individual organism. This permits the viewing and comparison of the temperature settings, on the growth of the test organisms, without the added confusion of the results from the other organisms. In all cases the numbers of colony forming units per mL are drastically reduced at 15°C. This difference was so marked with respect to *E. coli* and *P. aeruginosa* that magnifications of their results were produced and may be found in Figures 10 and 11. The fecal streptococci and *B. longum* seemed to suffer less from the reduced temperature of 15°C, rarely having CFU per mL enumerations which differed by even an order of magnitude.

4.2 Bactericidal effects of chlorine:

Tables 4 and 5 contain the results of chlorine exposure upon the test organisms *E. coli*, *P. aeruginosa*, *S. faecium* var. *casseliflavus* and *B. breve*. For all experimental situations, the exposure time to chlorine was for a 1 minute contact period. Table 4 is a record of the results obtained when the pH of the reaction flask was 6.0, while Table 5 illustrates the results obtained at pH 9.0.

The fact that the bactericidal effect of chlorine is not only exposure and concentration dependent becomes quickly apparent when the percentage of organisms killed is compared between the different pH settings. Also, the higher concentration of chlorine is much more lethal, at both pH settings, than is the 0.2 ppm concentration.

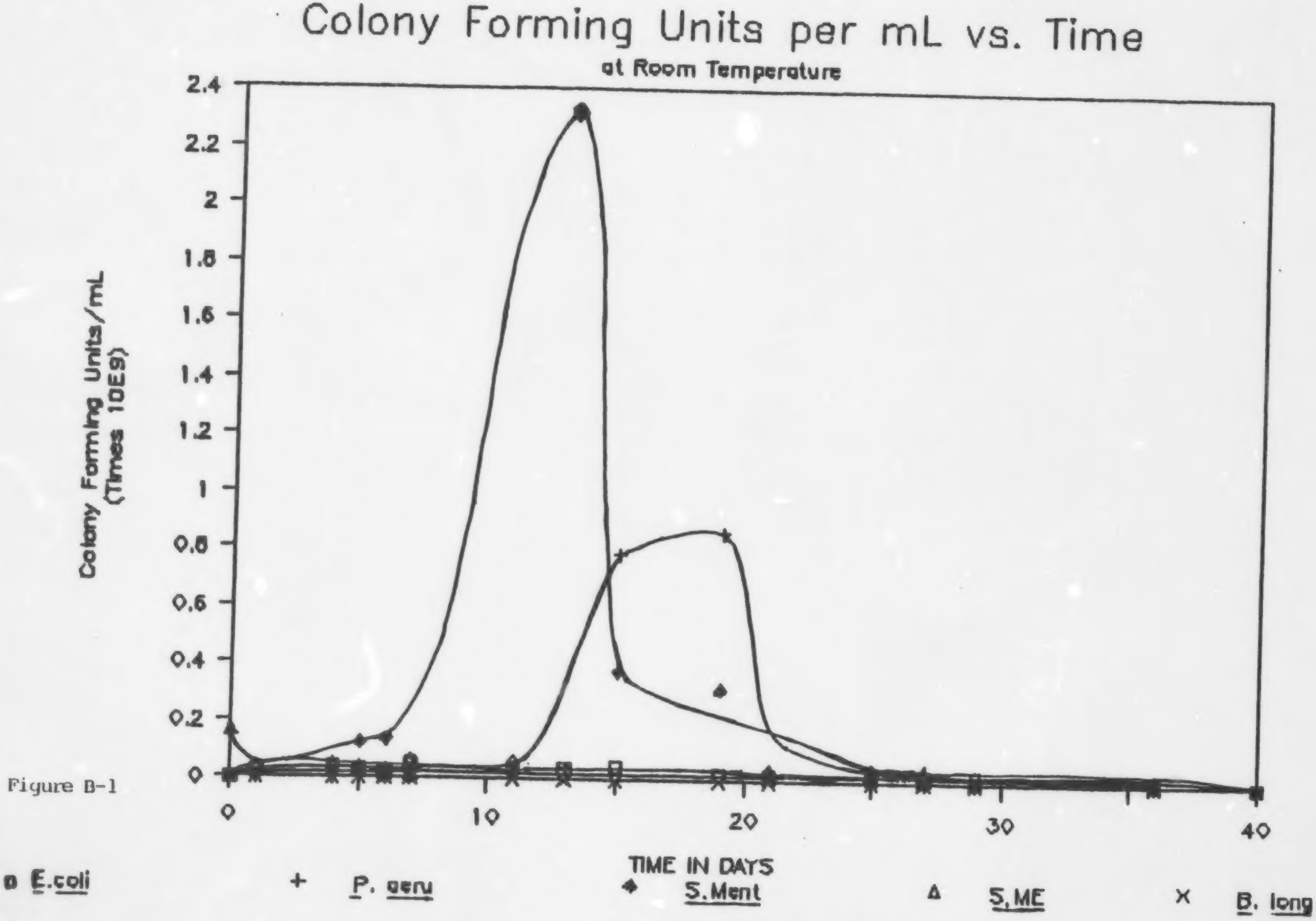

Colony Forming Units per mL vs. Time
at Room Temperature
Colony Forming Units/mL
(Times 10E9)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
TIME IN DAYS
□ E.coli
+ P. aeru
◆ S.Ment
Δ S.ME
× B. long

Figure B-1

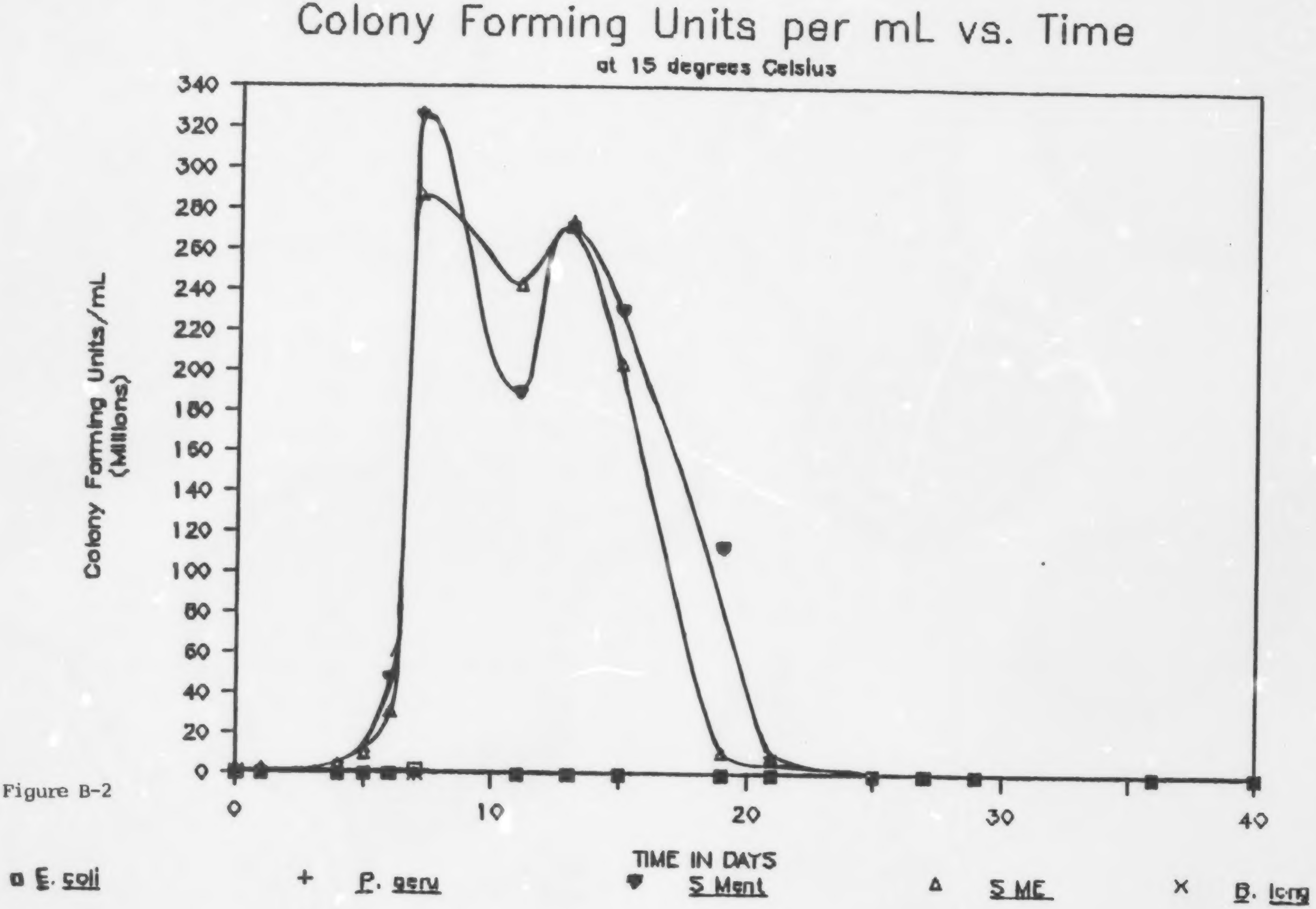

Colony Forming Units per mL vs. Time
at 15 degrees Celsius
Colony Forming Units/mL (Millions)
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
TIME IN DAYS
□ E. coli
+ P. aeru
▼ S Ment
△ S ME
× B. long

Figure B-2

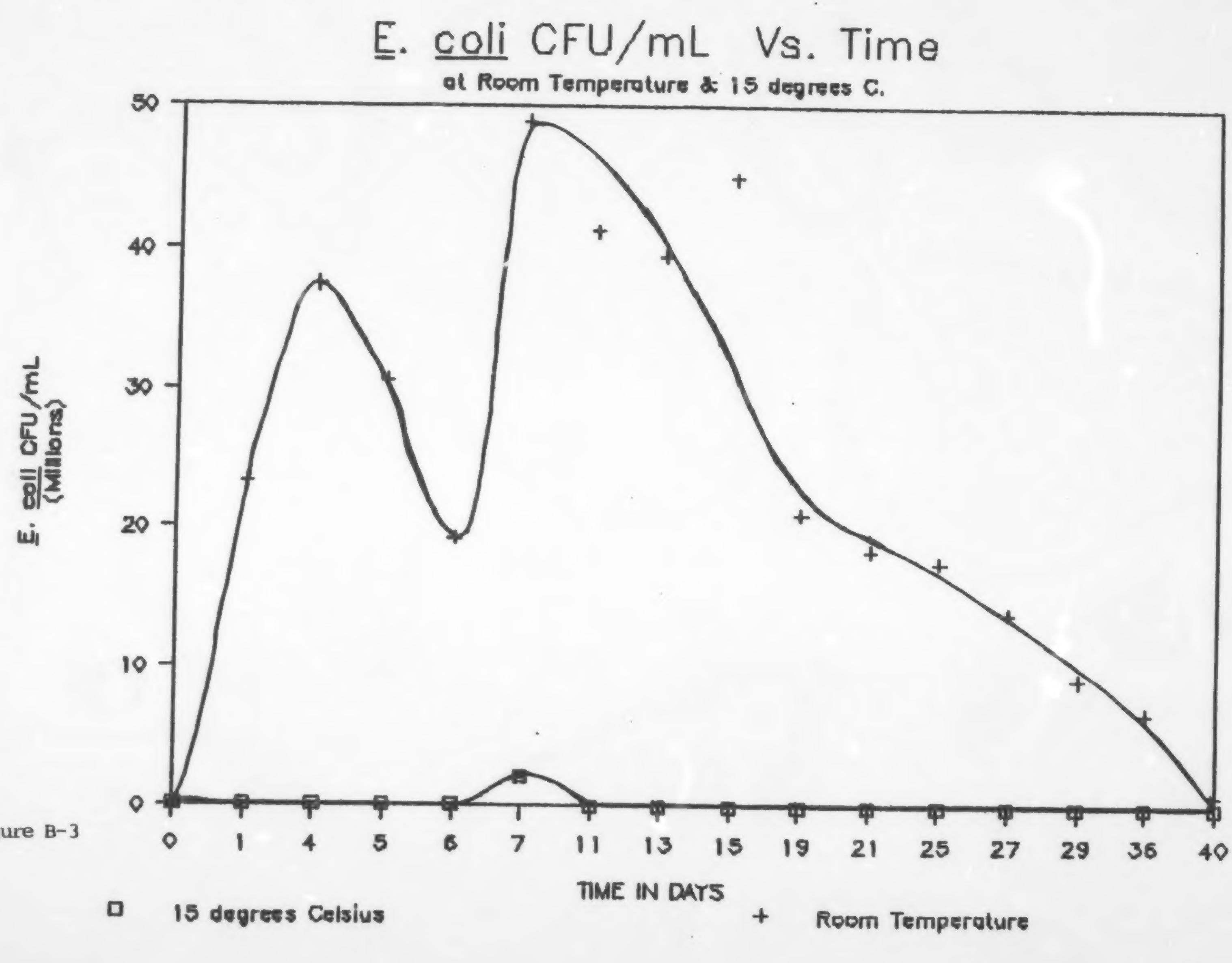

E. coli CFU/mL Vs. Time
at Room Temperature & 15 degrees C.
E. coli CFU/mL (Millions)
50
40
30
20
10
0
0
1
4
5
6
7
11
13
15
19
21
25
27
29
36
40
TIME IN DAYS
□ 15 degrees Celsius
+ Room Temperature

Figure B-3

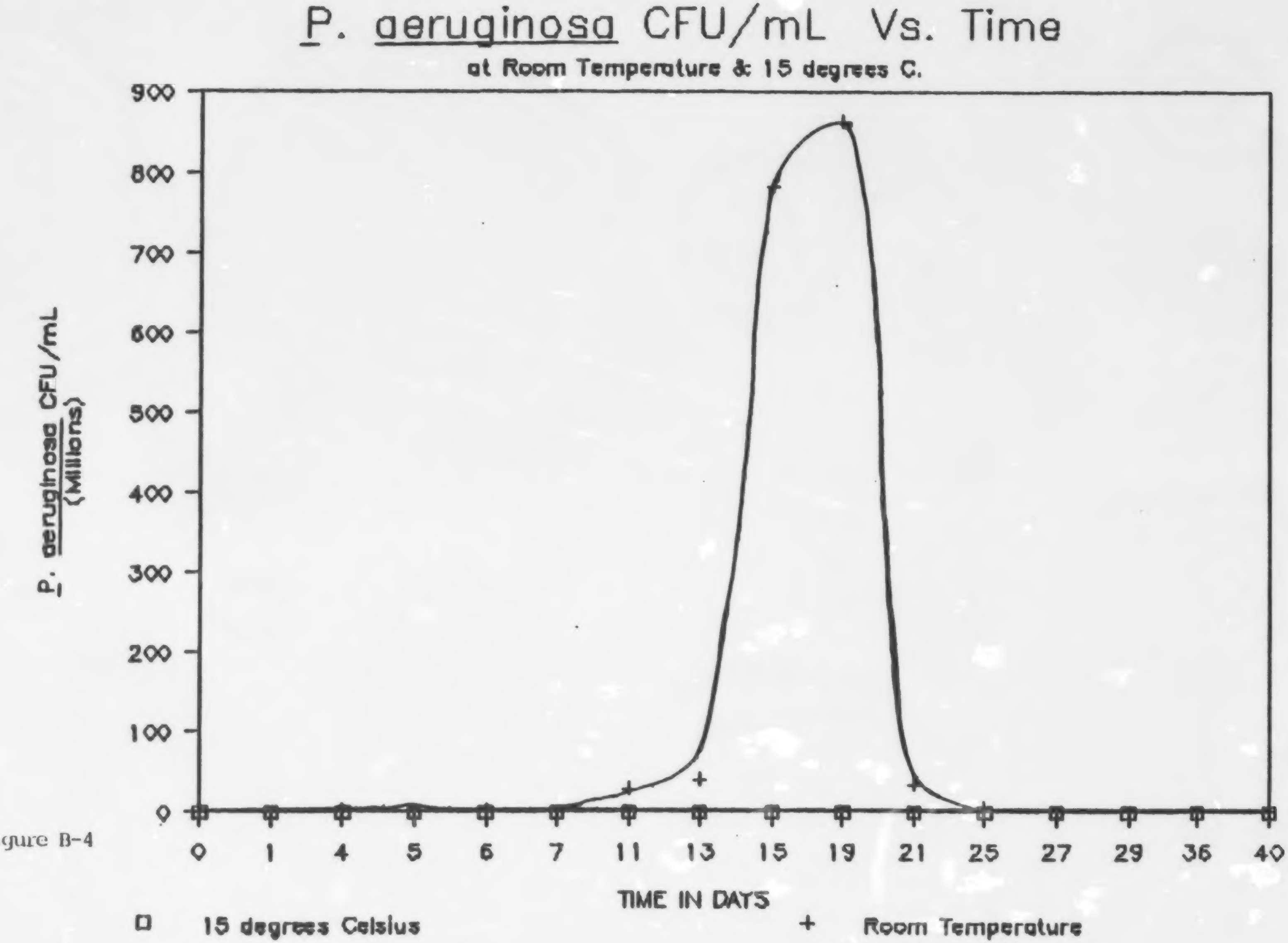

P. aeruginosa CFU/mL Vs. Time
at Room Temperature & 15 degrees C.
P. aeruginosa CFU/mL (Millions)
900
800
700
600
500
400
300
200
100
0
0
1
4
5
6
7
11
13
15
19
21
25
27
29
36
40
TIME IN DAYS
□ 15 degrees Celsius
+ Room Temperature

Figure B-4

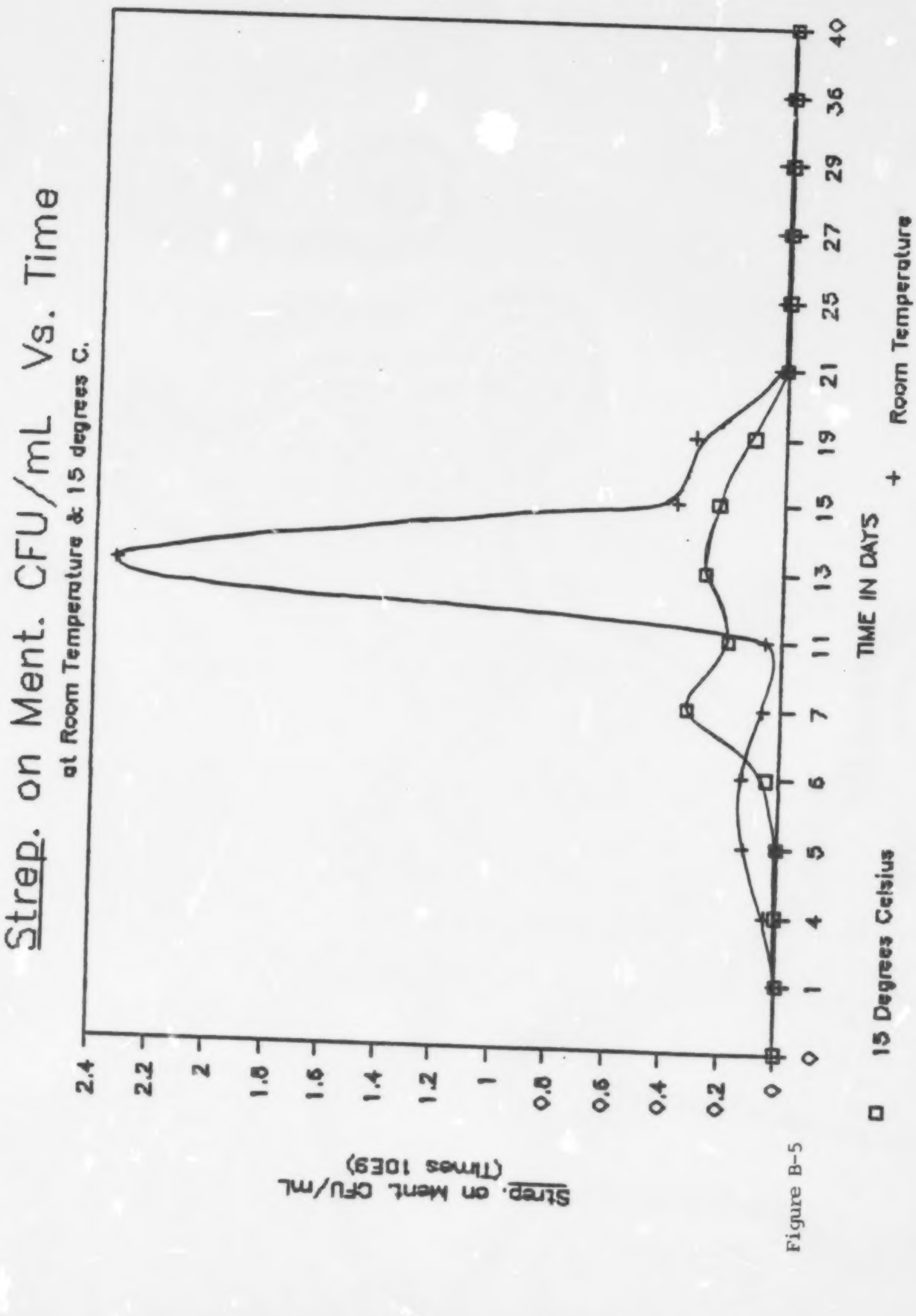

Strep. on Ment. CFU/mL Vs. Time
at Room Temperature & 15 degrees C.
Strep. on Ment. CFU/mL
(Times 10E9)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
4
5
6
7
11
13
15
19
21
25
27
29
36
40
TIME IN DAYS
□ 15 Degrees Celsius
+ Room Temperature

Figure B-5

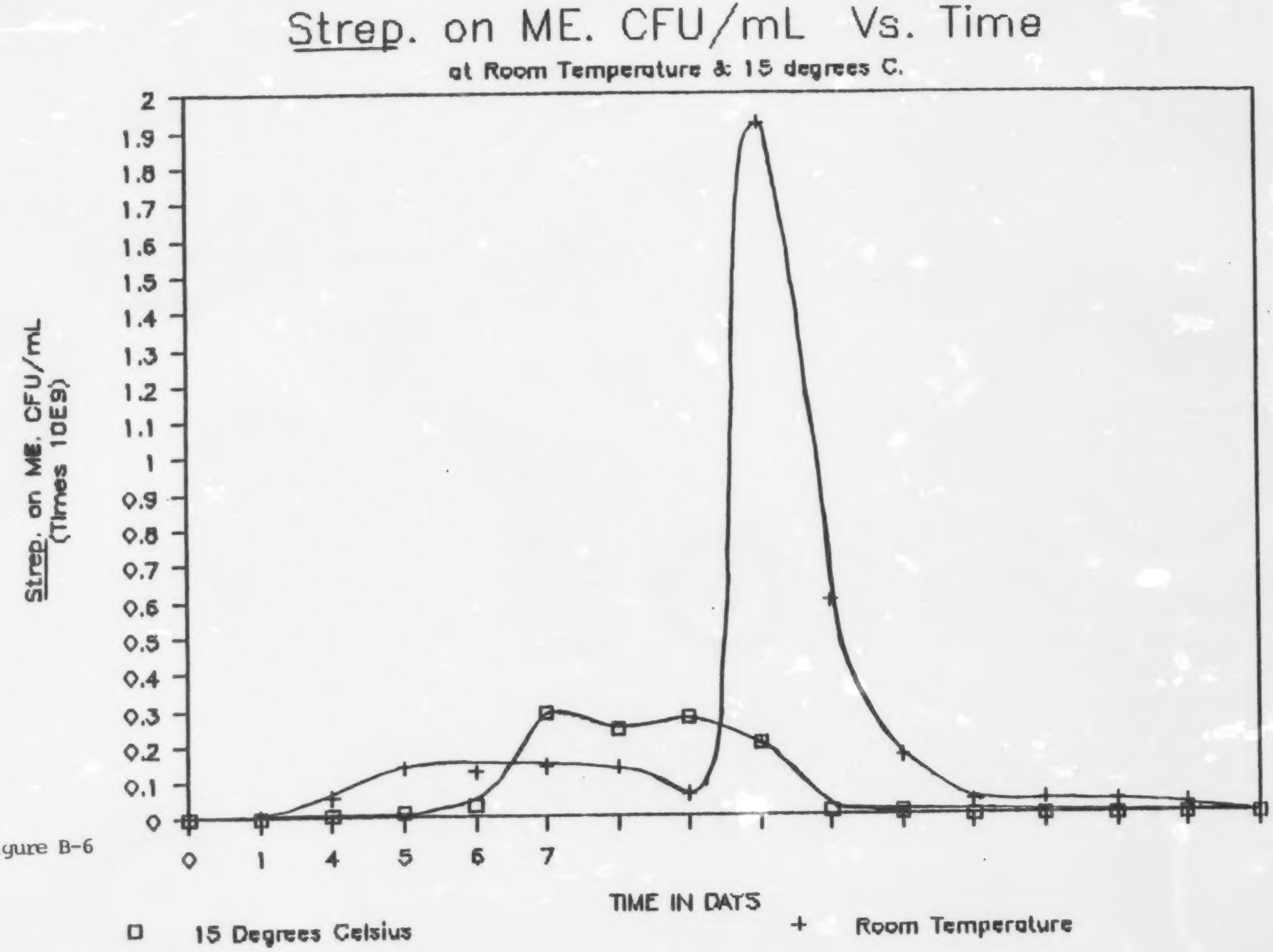

Strep. on ME. CFU/mL Vs. Time
at Room Temperature & 15 degrees C.
Strep. on ME. CFU/mL (Times 10E9)
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
4
5
6
7
TIME IN DAYS
□ 15 Degrees Celsius
+ Room Temperature

Figure B-6

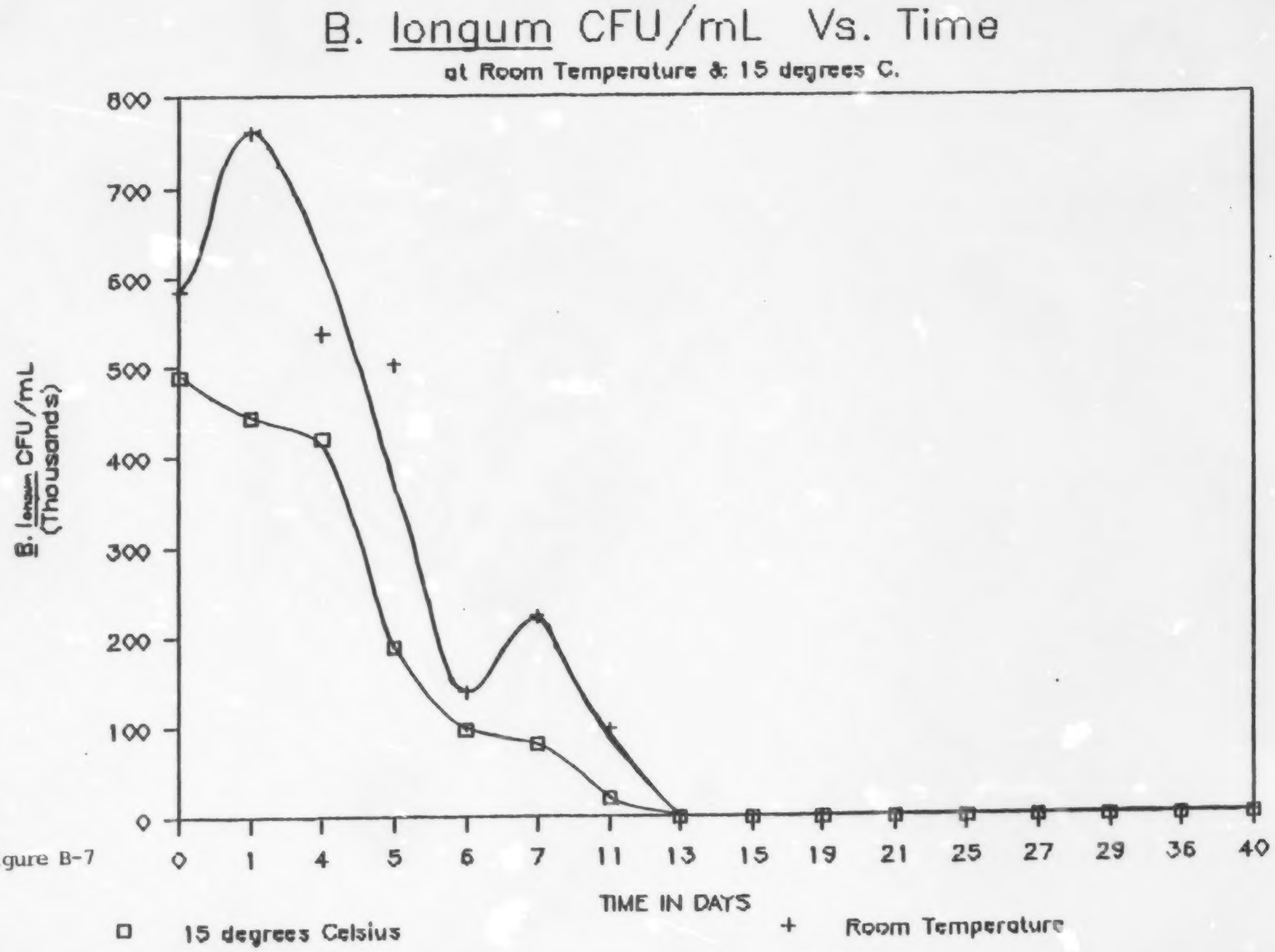
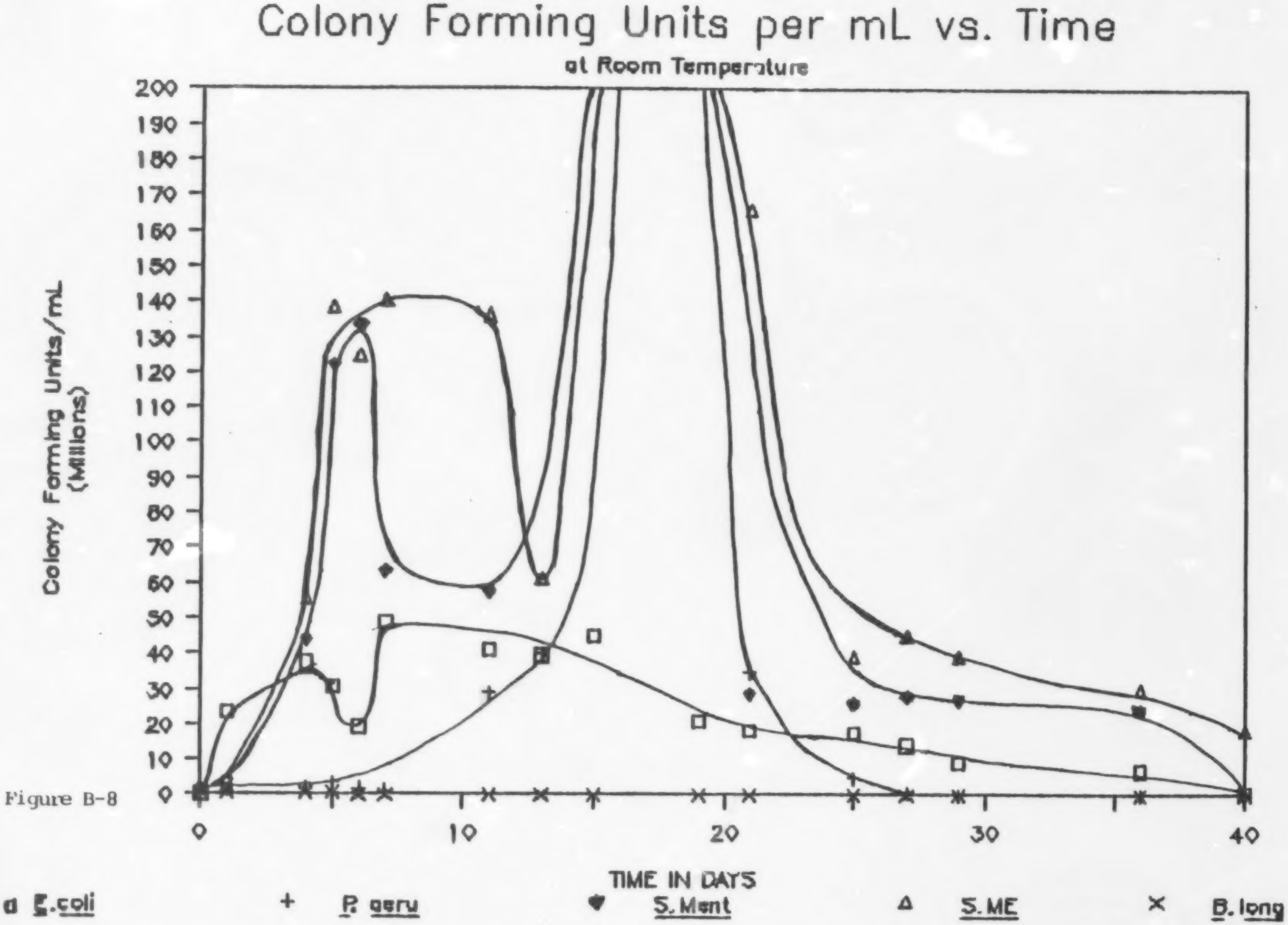

B. longum CFU/mL Vs. Time
at Room Temperature & 15 degrees C.
B. longum CFU/mL (Thousands)
800
700
600
500
400
300
200
100
0
0
1
4
5
6
7
11
13
15
19
21
25
27
29
36
40
TIME IN DAYS
□ 15 degrees Celsius
+ Room Temperature

Figure B-7



Colony Forming Units per mL vs. Time

at Room Temperature

Figure B-8

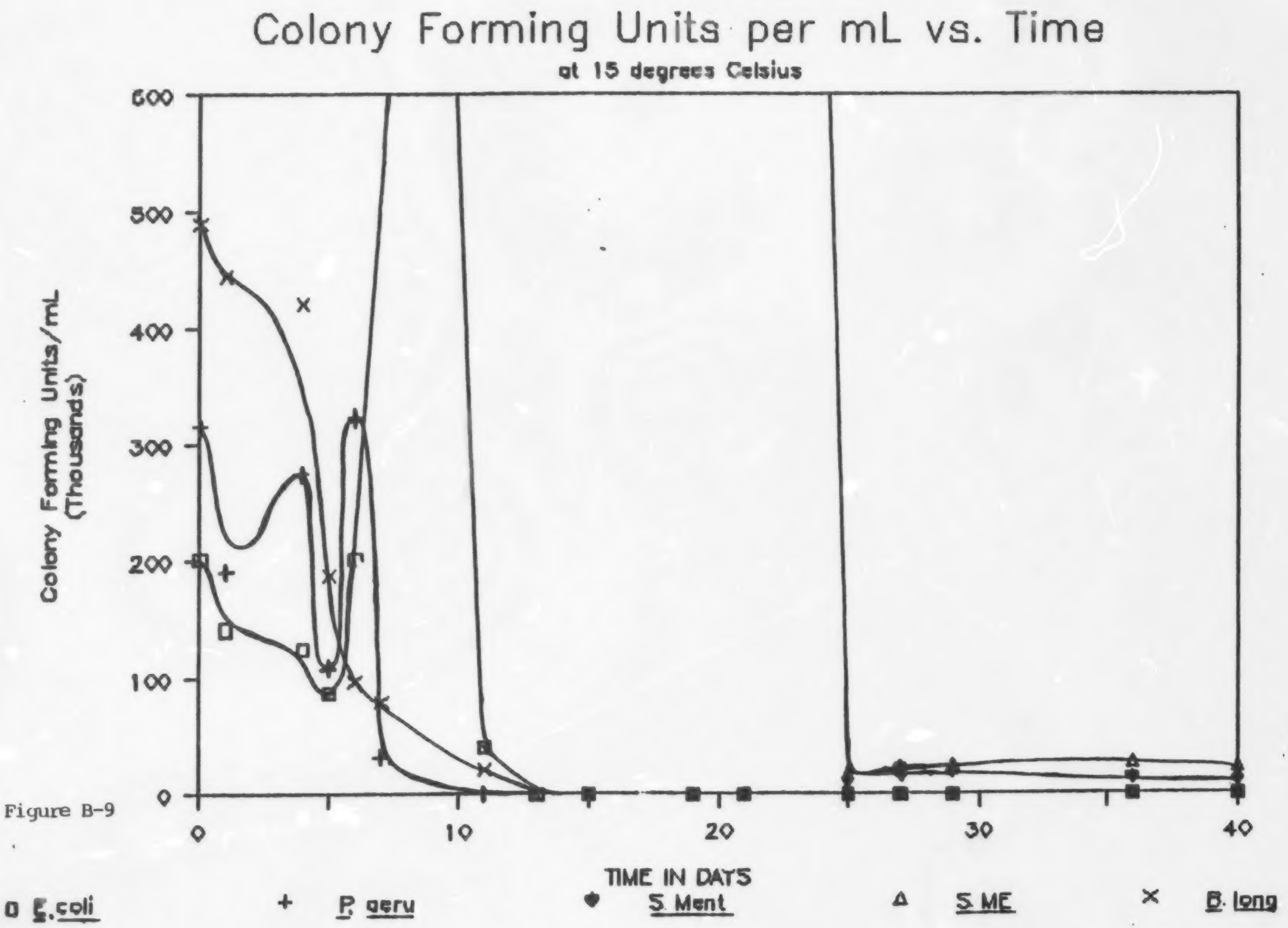

Colony Forming Units per mL vs. Time
at 15 degrees Celsius
Colony Forming Units/mL (Thousands)
600
500
400
300
200
100
0
0
10
20
30
40
TIME IN DAYS
□ E. coli
+ P. aeru
◆ S. Ment
△ S. ME
× B. long

Figure B-9

E. coli CFU/mL Vs. Time

at Room Temperature & 15 degrees C.

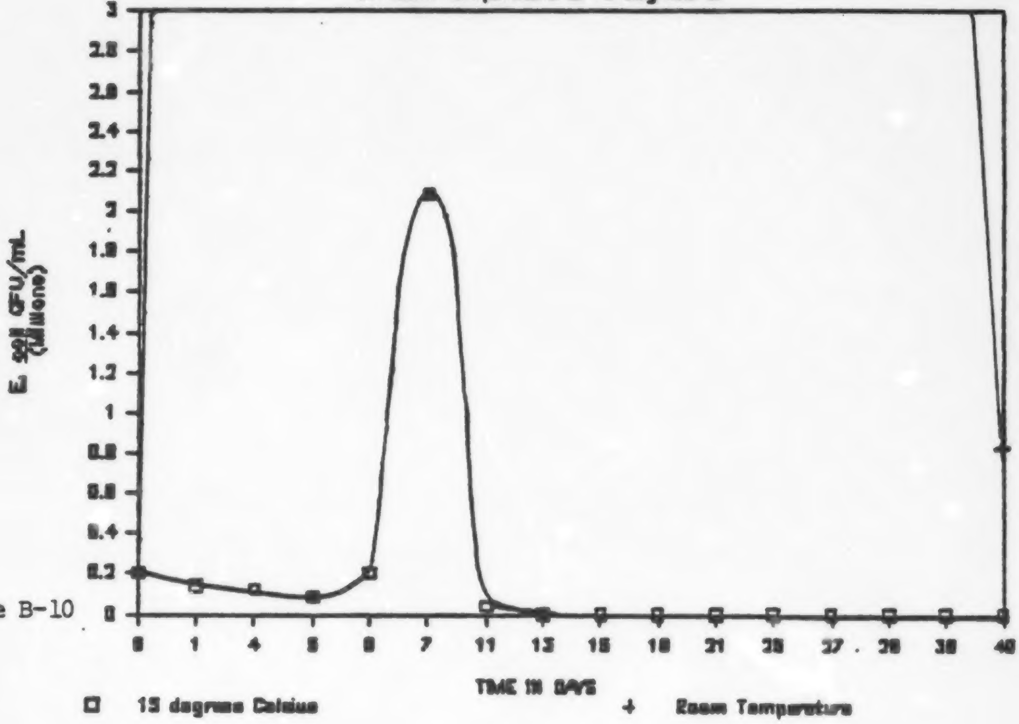


Figure B-10

P. aeruginosa CFU/mL Vs. Time

at Room Temperature & 15 degrees C.

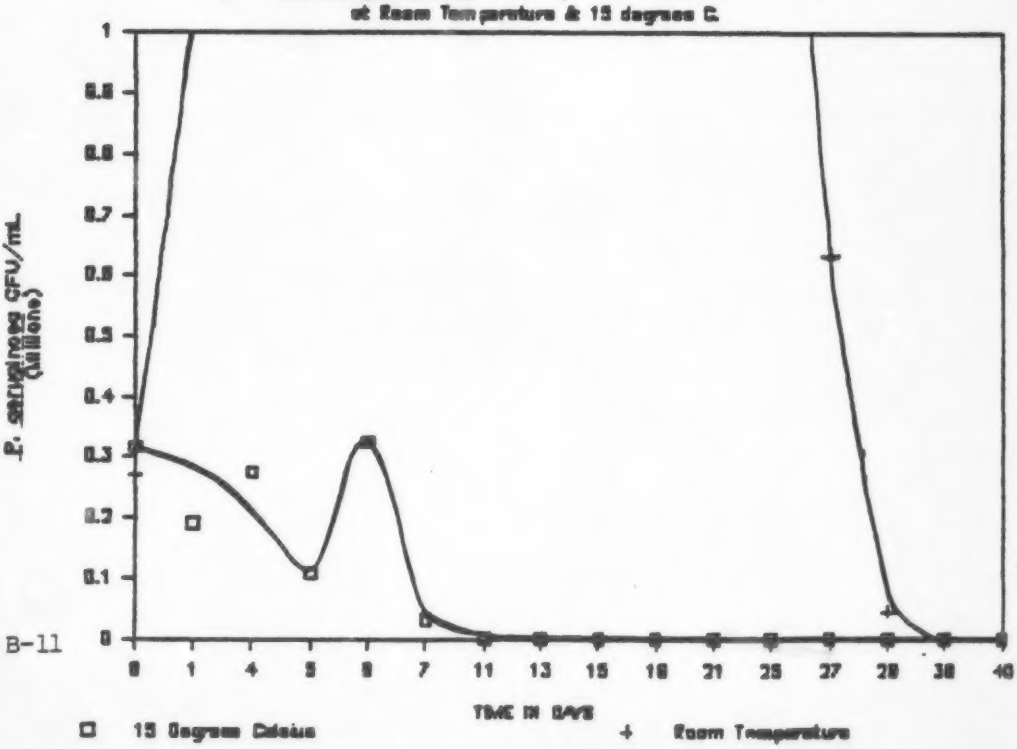


Figure B-11

One observation of particular interst is the tendency towards more effective killing, by chlorine, when it is functioning within an acidic environment. Only E. coli and S. faecium var. casseliflavus had a lower killing percentage (at 0.4 ppm) at the lower pH of 6.0. All other experimental determinations showed more effective killing at pH 6.0. This observation was particularly apparent in the killing of P. aeruginosa.

Figures 12 through 15 graphically depict analysis of the results of the chlorine experimentation. It is obvious that chlorine has lethal effects, from the negative slope of all the graphs, but more important is the drastic effect seen at 0.4 ppm in comparison to that of 0.2 ppm. At the two ends of the percentage killed spectrum lie E. coli, with the smallest percentage killed (0.2 ppm at pH at 9.0) and B. breve, experiencing the highest percentage killed (0.4 ppm at pH 6.0).

Table B-4

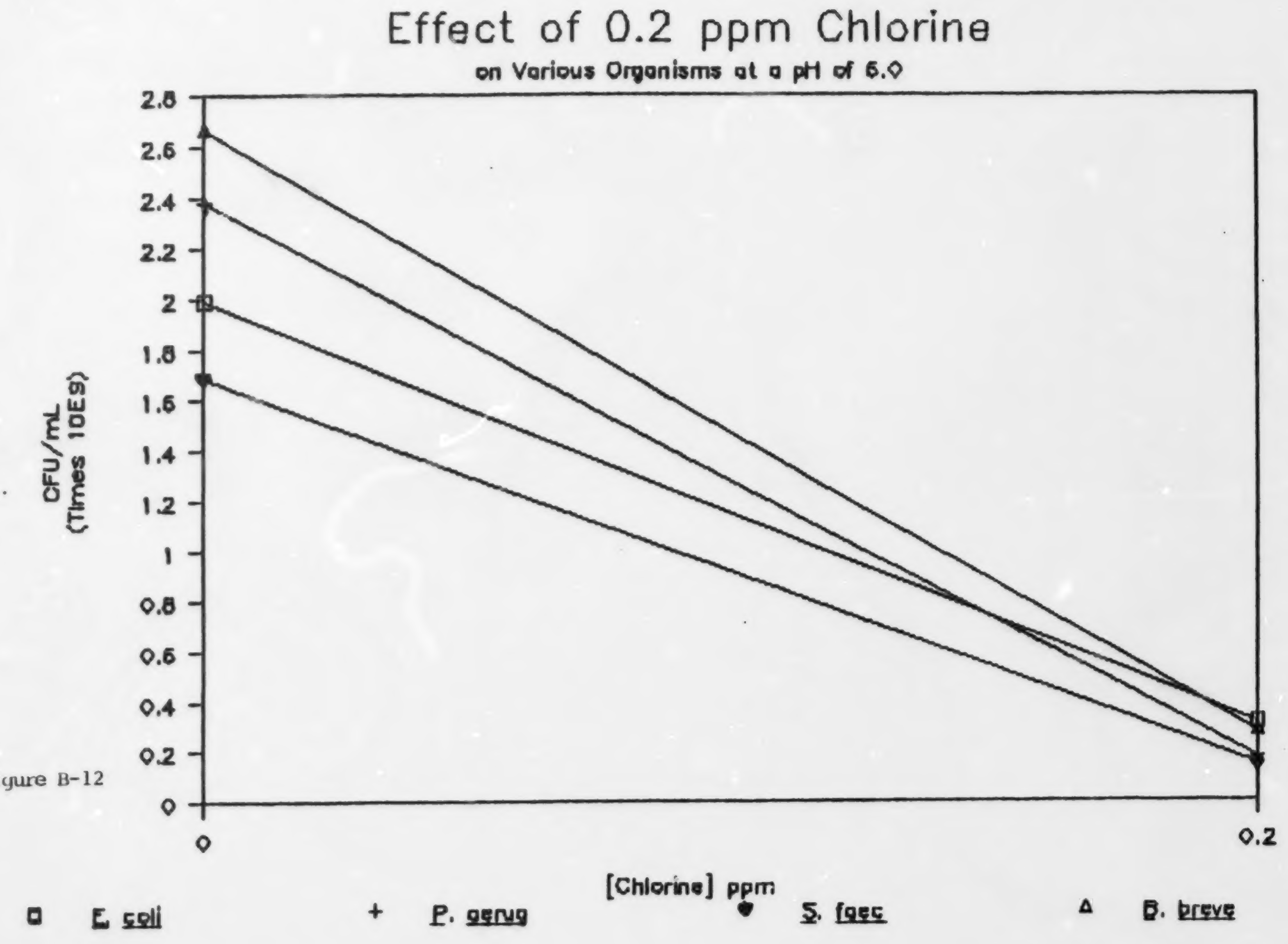
Effect of Chlorine at a pH of 6.0

ORGANISM	INITIAL CFU/mL	AFTER CHLORINE ADDITION		% KILLED	
		0.2 ppm	0.4 ppm	0.2 ppm	0.4 ppm
E. coli	1.99E+09	3.15E+08	1.31E+08	84.2	93.4
P. aeruginosa	2.38E+09	1.77E+08	8.25E+07	92.6	96.5
S. faecium var. casseliflavus	1.69E+09	1.52E+08	8.65E+07	91	94.9
B. breve	2.67E+09	2.82E+08	5.40E+07	89.4	98

Table B-5

Effect of Chlorine at a pH of 9.0

ORGANISM	INITIAL CFU/mL	AFTER CHLORINE ADDITION		% KILLED	
		0.2 ppm	0.4 ppm	0.2 ppm	0.4 ppm
E. coli	3.80E+09	1.08E+09	2.30E+08	71.6	93.9
P. aeruginosa	1.82E+09	3.47E+08	1.90E+08	80.9	89.6
S. faecium var. casseliflavus	2.80E+09	2.73E+08	1.20E+08	90.3	95.7
B. breve	6.15E+09	1.04E+09	3.16E+08	83.1	94.9



Effect of 0.2 ppm Chlorine

on Various Organisms at a pH of 6.0

Figure B-12

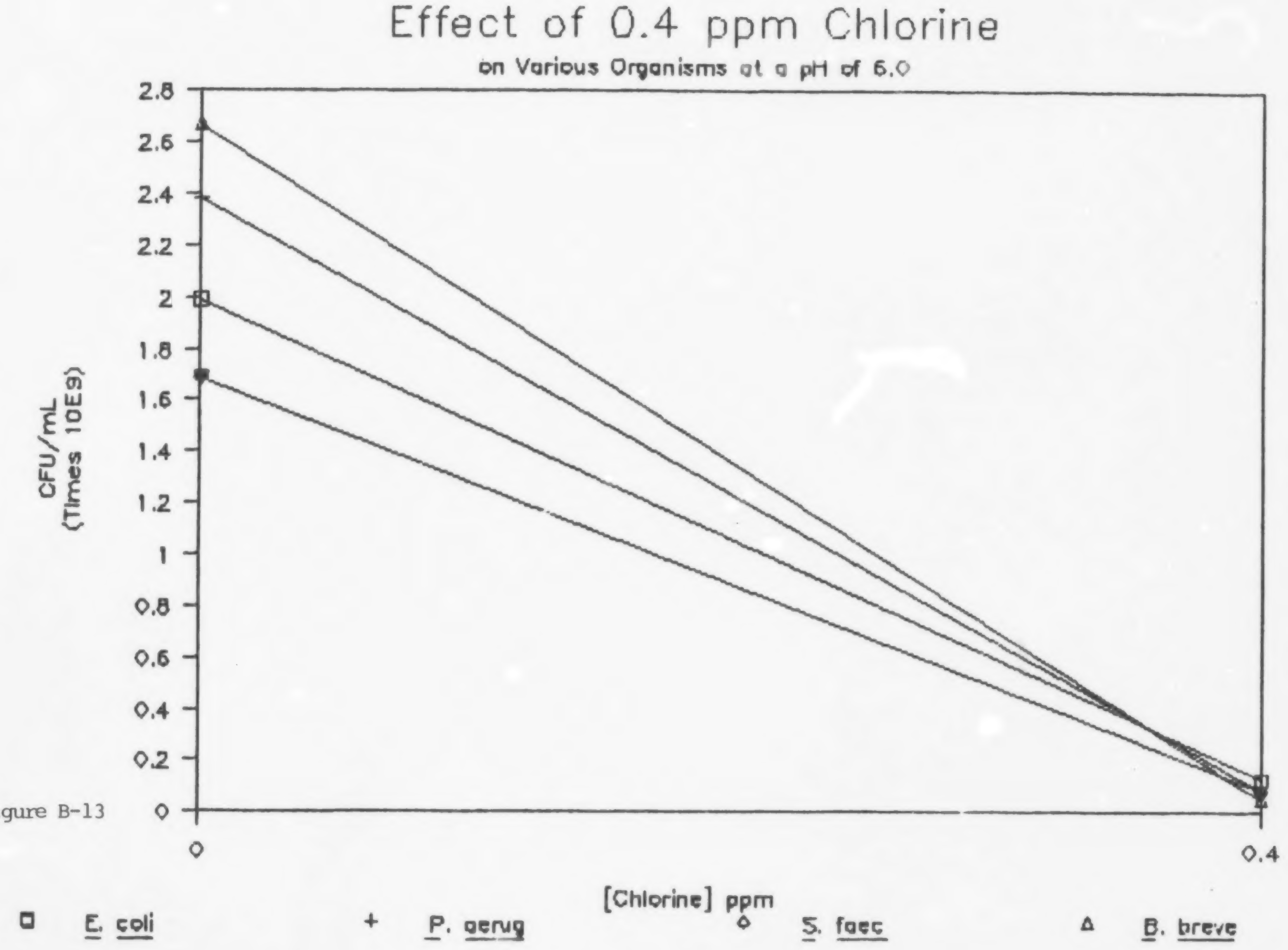

Effect of 0.4 ppm Chlorine
on Various Organisms at a pH of 6.0
CFU/mL
(Times 10E9)
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.4
[Chlorine] ppm
□ E. coli
+ P. aerug
◇ S. faec
△ B. breve

Figure B-13

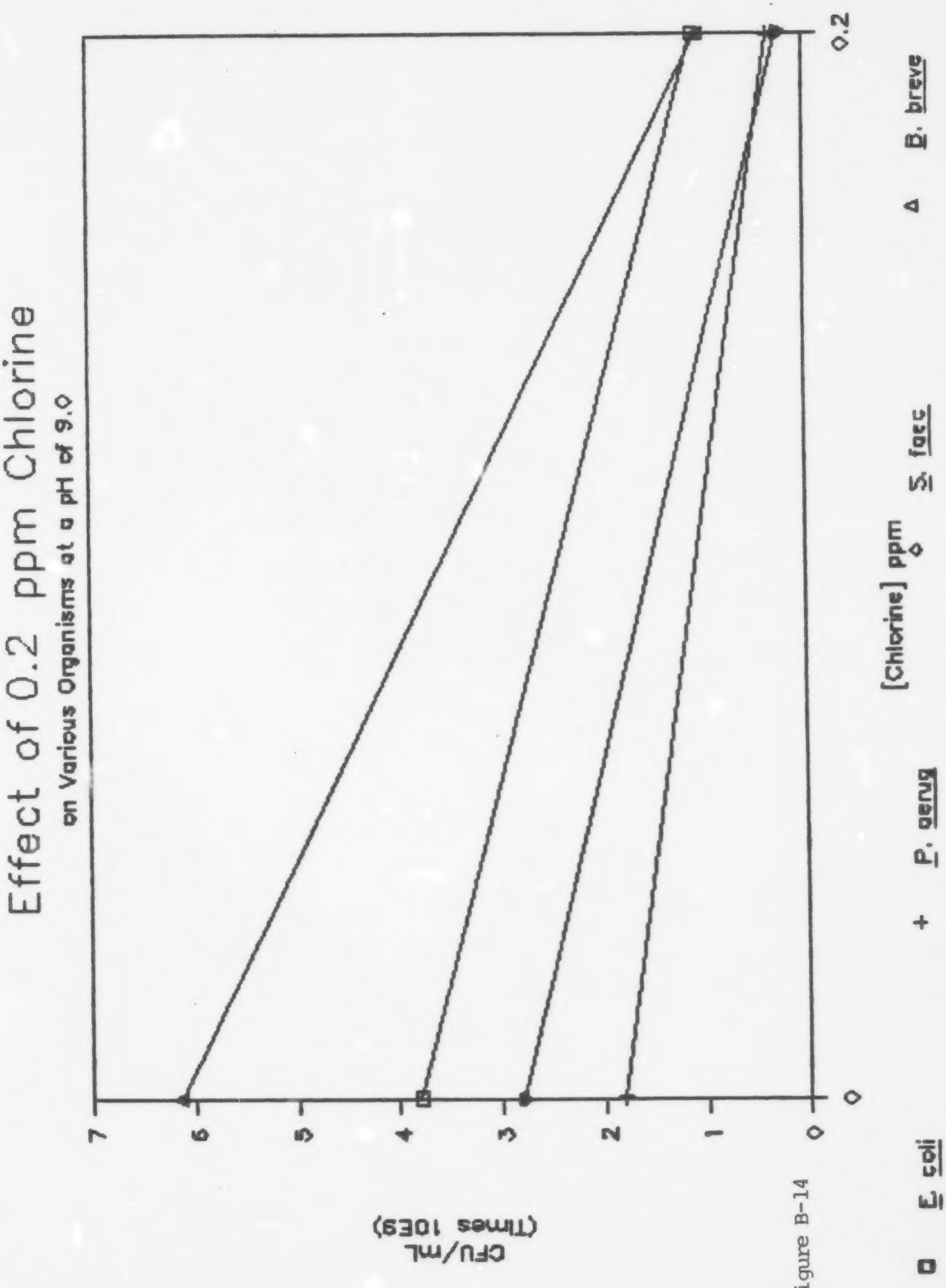

Effect of 0.2 ppm Chlorine
on Various Organisms at a pH of 9.0
CFU/mL
(Times 10E9)
0
1
2
3
4
5
6
7
0
0.2
[Chlorine] ppm
□ E. coli
+ P. aerug
◇ S. faec
△ B. breve

Figure B-14

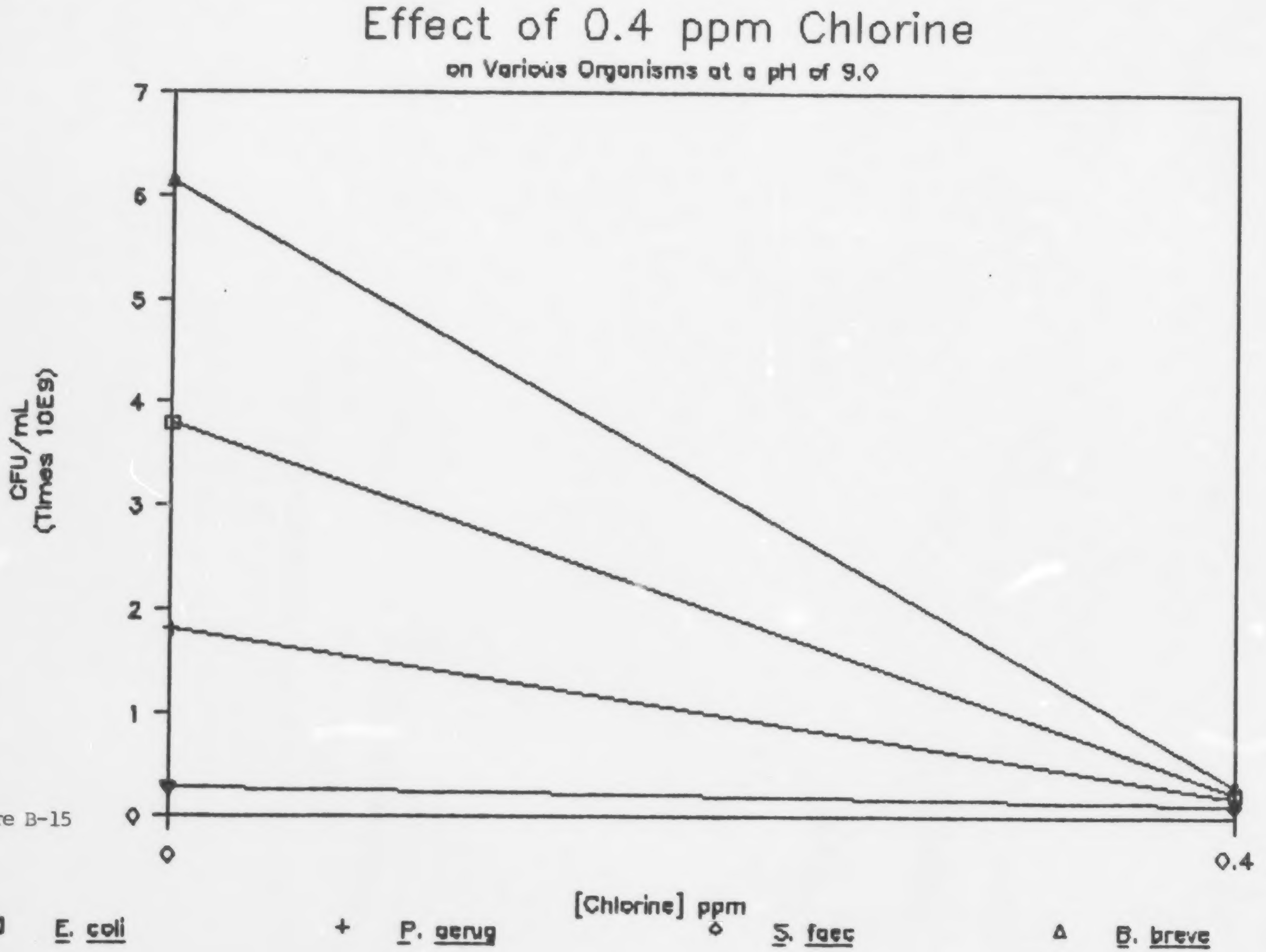

Effect of 0.4 ppm Chlorine
on Various Organisms at a pH of 9.0
CFU/mL
(Times 10E9)
7
6
5
4
3
2
1
0
0
0.4
[Chlorine] ppm
□ E. coli
+ P. aerug
◇ S. faec
△ B. breve

Figure B-15

DISCUSSION

The analysis of the growth cycles of E. coli, P. aeruginosa, S. faecalis var. faecalis, S. faecium and B. longum had as its goal the development of a methodology for the purpose of locating a bacterial indicator which could be used for the detection of recent fecal contamination within surface water bodies. This is a problem of growing public concern, since the potential health hazard such pollution creates is of grave importance. The experimental trial at room temperature served to act as a standard or means of comparison for those results obtained at 15°C. This temperature was selected to simulate, on the basis of temperature alone, the environment of a storm water sewer (Seyfried personal communication, 1988). It has been reported by the Toronto Area Watershed Management (TAWM) that a major contributor to fecal contamination within the Humber and Don Rivers is the effluent from Toronto storm water sewers.

The controversy over which bacterial indicator to use, for the purpose of water pollution control policies, has resulted in much debate. Taylor et al. (1973), state that the enumeration of fecal coliforms provides a more precise measure of the potential health hazard in surface water bodies, than does the more omniscient total coliforms. According to Doran and Linn (1979) fecal coliforms are reported to be the most reliable indicator of fecal pollution of water; however, with these organisms the source of contamination may not be identified. To compensate for this the use of fecal streptococci has been suggested in order to differentiate fecal contaminants from human or other animal sources (Geldrich and Kenner, 1969). Schuettpelz (1969) reports that enterococci approach numbers of total coliforms in sewage, but have the added advantage of not multiplying in water.

It would appear that the trend to employ fecal streptococci as indicators of fecal pollution is growing, but problems with their acceptance still exist.

Firstly, fecal streptococci are present in smaller numbers in feces, sewage and polluted waters, and the easier quantification of fecal coliforms is inhibitory to their use. The second major problem lies within the confusion which exists concerning the identity of fecal streptococci, in particular, that of their ecological distribution (Levin et al. 1975). To overcome this handicap a barrage of research has been performed in the field of developing media selective for specific fecal streptococci, so that their identity may be positively confirmed. Work done by Isenberg et al. (1970), Wie-Shing Lee (1972), Switzer and Evans (1974) and Brodsky and Schiemann (1976) is illustrative of only a minor sampling of involved participants. Another attempt at overcoming the barrier of being unable to identify the source of the fecal contamination has been to use the fecal coliform (FC) to fecal streptococci (FS) ratio. Doran and Linn (1979) claim that an FC/FS ratio of greater than four is usually indicative of domestic waste water pollution, while an FC/FS ratio of less than 0.7 may be associated with non-human animal wastes.

Recently, P. aeruginosa has been considered as a potential indicator of fecal contamination from human sources. Wheater and co-workers (1978) have shown that P. aeruginosa was not found in a variety of animal feces and also that the presence of this organism in animals and soil was due to chance and the close proximity to man (approximately 15% of the human population contain P. aeruginosa as a normal commensal microbe). For this reason and because of the opportunistic pathogenic nature of P. aeruginosa it has been given consideration as an indicator of fecal contamination in surface water bodies.

The final organism, B. longum, which was included in this growth study comparison is a member of a genus upon which a great deal of research has not been done. Classification and identification of Bifidobacterium species monopolizes the research of this organism. Its membership as an intestinal bacteria and subsequently as a fecal organism prompted its inclusion.

Concerning the 15°C temperature setting, experimental results showed that P. aeruginosa had the shortest life span. As well, E. coli (fecal coliform) and B. longum have life expectancies only slightly longer than that of P. aeruginosa. The two fecal streptococci survived longest, and were still growing when experimentation was terminated. The problem encountered here was that the continued growth of both S. faecalis var. faecalis and S. faecium prevented the determination of which organism died first. Biochemical testing done at Day 30 on the fecal streptococci resulted in the identification of both species, with continued "picking" being unfeasible because of time limitations.

Despite the stated goal of this reasearch, namely the determination of a bacterial indicator of recent fecal contamination of surface water bodies, the theoretical approach was to simulate an environment like that of a storm sewer, since these conduits act as a major vehicle for the transport of fecal pollutants. In this manner the source of the contamination is addressed, as opposed to identifying and then attempting to treat the surface water bodies which have been defiled. E. coli, P. aeruginosa and B. longum seem to all possess the criteria of having a short life. Therefore, for purposes of identifying the newness of fecal contaminants they could feasibly be employed. The experimental data collected indicates this conclusion, however, environmental concern regarding this topic does not end with simply identifying how recent the pollution is, it also requires the differentiation of human and animal fecal contamination. The human input into the problem of surface water pollution may, with perseverance and regulation, be controlled; however, for the animal contribution this task would not be practical. Thus it becomes essential to discriminate between human and animal fecal waste, which as per the literature seems to necessitate the use of identifiable fecal streptococci.

The second portion of this research study was involved with examining the bactericidal effect of chlorine upon E. coli, P. aeruginosa, S. faecium var. casseliflavus and B. breve. The wide acceptance of chlorine for purposes of disinfection stimulated interest in whether or not chlorine, under appropriate conditions could be used as a sterilant. The fact that this research project began as a survival study must also not be overlooked. The two parameters which were given consideration in this study included the pH of the environment in which the chlorine functioned, as well as the particular concentration of the chlorine exposure dosage. The length of application of chlorine was not manipulated, being kept constant at one minute for all trials.

According to Camper and McFeters (1979) exposure of waterborne organisms of fecal origin to hostile chemical environments results in a chain of events. The first of these is stress, which is then followed by injury and finally, death. If microorganisms have been exposed to chlorine, as in this experimentation, then the cultivation of survivors necessitates the use of a non-selective (not stressful) growth medium. For this reason, the antibiotic free nutrient, BHI, and MRS agars were used. In some cases it has been suggested that the injuries sustained due to chlorine may be reversed with the addition of appropriate metabolites to the growth medium (Heinmets et al., 1954).

With reference to the Introduction, the biochemical effect of chlorine upon the bacterial cell may be found. One specific mode of operation, which was not mentioned previously, comes from the early work of Knox, et al. (1948) in which it was proposed that chlorine specifically oxidizes sulfhydryl groups of certain enzymes important in carbohydrate metabolism. These hypothetical models of chlorine action provide a basis from which the physiologic understanding of this chemical's function may be extrapolated. This knowledge is essential if chlorine is to be considered for purposes of sterilization, since its limitations must be known.

Experimentation with chlorine requires the use of acid-washed glassware, chlorine demand-free test waters and bacteria free of chlorine-demand products. Fitzgerald and Dervartanian (1969) presented experimental data, regarding P. aeruginosa, which illustrated why such materials are of paramount importance. An unwashed suspension of P. aeruginosa (concentration of 10E6) had a chlorine demand of 0.4 ppm while washed bacteria had a demand of one-tenth this amount. Some examples of chlorine-demanding products may again be found within the Introduction. The reason for their removal prior to experimentation resides within their ability to stabilize chlorine and render it nearly functionless.

One final point concerning the operation of chlorine deals with the kinetics and the more general functioning of this bactericidal agent. Three basic factors influence chlorine's effect on the bacterial cell. The first of these is the mass transfer of chlorine to the bacterial cell liquid interface. Secondly, the chemisorption of the chlorine at selective centres on the cell surface and finally, "the surface and intrasurface diffusion of the activated chemisorbed complex with attendant chemical attack on cellular elements" (Bernarde et al., 1967).

This schemata illustrates the "gross" functioning of chlorine, as well, a feel for the numerous locations for rate limiting steps may be obtained.

The experimental results obtained through this research project (see Results) seemed to correlate, with only two trial exceptions, to the theoretical concepts presented by Black et al. (1970), in which it was reported that the bactericidal effectiveness of chlorine was greater at an acidic pH. The implications here are that the hypochlorite ion is not as effective a disinfectant as hydrochlorous acid, which referring to Table 7, makes the adjustment of the pH a prerequisite for adequate killing.

Table 6

Effect of pH on forms of Chlorine

pH	% Cl	% HOCl	% OCl
4	0.5	99.5	0
5	0	99.5	0.5
6	0	96.5	3.5
7	0	72.5	27.5
8	0	21.5	78.5
9	0	1	99

(Black et al., 1970)

Further examination of the results shows that at higher concentrations the percentage of organisms killed was increased. The use of chlorine as a sterilant, however, is questionable. Neither the acidic conditions nor the increased concentration of 0.4 ppm (theoretically ideal situation) provided 100% killing, demanded of an agent used for sterilization. *B. breve* was the only organism with a percentage killed close to that of sterility, and it was yet 2 percentage points short. Further increase in chlorine concentration, coupled with an exposure time of greater than on minute may be needed to attain sterile conditions.

The final aspect of the chlorine experimentation deals with the ability of some organisms to resist the lethal effects of this chemical. Four main factors are associated with this resistance and they include: (i) cell surface structure modification which may facilitate cell clumping, (ii) bacterial adhesion to suspended particulate matter (clay particles), (iii) production of extracellular capsules or slime layers and (iv) formation of resistant spores (Ridgway and Olson, 1982). From the test organisms used, P. aeruginosa was the only one with the ability to produce a slime layer which according to Brown (1975) may act by attaching chemicals and preventing their penetration into the cell. The experimental results in a pH of 9.0 do, for P. aeruginosa, indicate a lower percentage killed than the other organisms, however, this was not the case at a pH of 6.0. P. aeruginosa's greater resistance may also be attributed to this organisms tendency to form cell aggregations.

RECOMMENDATIONS

6.1 Growth cycle determination

- To facilitate the study of the fecal streptococci a superior approach may be to combine *E. coli*, *P. aeruginosa*, *B. longum* and the first case *S. faecalis* var. *faecalis* and in the second case *S. faecium*.
- The second alteration would be to study the growth of the organisms in an open-system, as opposed to the closed environment used in this study.

6.2 Chlorine testing

- The bactericidal effect of chlorine could be tested on more resistant organisms, such as the *Mycoplasma* or endospore forming organisms.
- Further experimentation with the manipultion of the time of exposure to chlorine, in conjunction with a wider range of chlorine concentration.

Appendix

7.1 Buffers and Solutions:

1) - Calcium hypochlorite (stock) solution:

Ca(OC)	0.5g
dH_2O	500mL

Stir ingredients to dissolve. Refridgerate in the dark.

2) - 1.0M Hydrochloric acid:

BDH Analar grade concentrated HCl	41.5mL
dH_2O	500mL

Add concentrated HCl to ~200mL dH_2O and then add the remaining 300mL dH_2O. Dispense into bottles and autoclave for 15 minutes at 121°C / 15 PSI. Keep refridgerated.

3) - Phosphate solution:

(A) Dissolve 34.0g KH_2PO_4 in 500mL dH_2O.
Adjust pH to 7.2
Dilute to 1 Litre with dH_2O

(B) Dissolve 50g $MgSO_4 \cdot 7H_2O$ in 1 Litre dH_2O.

Autoclave both solutions separately for 15 minutes at 121°C / 15 PSI. Cool and store for up to 1 month. Add 1.25mL of (A) and 5mL of (B) to 1 Litre of dH_2O. Dispense as dilution blanks or for rinse water. Autoclave for 15 minutes at 121°C / 15 PSI.

4) - Sodium thiosulfate solution:

$Na_2S_2O_3$	0.35g
dH_2O	500mL

Stir ingredients to dissolve. Add 5mL of $Na_2S_2O_3$ to acid washed test tubes. Autoclave for 15 minutes at 121°C / 15 PSI.

5) - 1.0M Sodium hydroxide:

NaOH	40.0g
dH_2O	100mL

Dissolve NaOH in dH_2O slowly. Dispense into bottles and autoclave for 15 minutes at 121°C / 15 PSI.

7.2 Growth Media:

The following media was prepared according to manufacturer's specifications:

DIFCO media:

1) - BHI agar

2) - Nutrient agar 1.5% (NA) 3) -Nutrient broth

4) - Sugars for biochemical testing, prepared to a final concentration of 0.5%.

- Arabinose
- Lactose
- Mannitol
- Melelitose
- Melibiose
- Cellobiose
- Raffinose
- Ribose
- Sorbose
- Trehalose
- Xylose

5) - The remainder of the biochemical testing agents were also prepared to manufacturer's specifications:

- Argenine
- Bile esculin
- Gelatin
- Litmus milk
- Kligler's
- Pyruvate
- Sodium chloride
- Todd Hewitt

GIBCO media

6) - MRS broth

7) - MRS agar - with the addition of 0.03% cysteine hydrochloride

8) - M-Enterococcus agar (m-Ent):

M-Enterococcus agar (DIFCO)	42g
*Sterile dH_2	1000mL

*Autoclave distilled water first, prior to making media. Allow to cool. Weigh out agar in a sterile beaker using an alcohol flamed spatula. Heat to dissolve agar (93°C). Cool rapidly to 60°C and dispense into square petri plates. Final pH 7.2 +/- 0.2.

9) - m-E medium (Dufour's modified):

- Peptone	10.0g
- Yeast extract	30.0g
- Sodium chloride	15.0g
- Sodium azide	0.15g
- Actidione	0.05g
- Agar	15.0g
- dH_2O	1000mL

Mix above ingredients and heat to 90°C. Autoclave for 15 minutes at 121°C / 15 PSI. Cool medium to 60°C and stir in *Antibiotic solution. Dispense into square sterile petri dishes. Store at 4°C after solidification. Final pH 7.1 +/- 0.1.

*Antibiotic solution:

Add each separately, asceptically

A) Nalidixic acid	- 0.240g in 3mL dH_2O and 0.2mL 10M NaOH

Add last:

B) Triphenyltetrazolium chloride - 0.020g

C) Indoxyl - B - D glucose - 0.500g in 5mL 95% ethanol and 5mL dH_2O

10) - Medium for Pseudomonas aeruginosa (MPA):

- L - lysine monohydroechoride	5.0g
- Yeast extract	2.0g
- Xylose	2.5g
- Sodium thiosulphate	5.0g
- Magnesium sulphate, anhydrous	1.5g
- Sucrose	1.25g
- Lactose	1.25g
- Sodium chloride	5.0g
- Ferric ammonium citrate	0.80g
- Sodium desoxycholate	0.10g
- Phenol red	0.08g
- dH_2O sterile	800mL

Autoclave 800 mL distilled water before preparing media.

Mix above ingredients and adjust pH to 7.6. Add 15g agar. Heat to 93°C to dissolve agar and then cool to 60°C. Stir in *Antibiotic solution. Dispense into square sterile petri dishes. Store at 4°C after solidification. Final pH 7.1 +/- 0.1.

*Antibiotic solution:

A) Sulfapyridine	0.1760g
B) Kanamycin sulphate	0.0085g
C) Naladixic acid	0.0370g
D) Actidione	0.1500g

Dissolve A to D in 200mL sterile dH_2O. Heat to 50°C to dissolve antibiotics.

11) - Medium for the Isolation of Thermo Tolerant E. coli

(M-Tec agar):

- Proteose peptone No. 3	5.0g
- Yeast extract	3.0g
- Lactose	10.0g
- NaCl	7.5g
- K_2HPO_4	3.3g
- KH_2PO_4	1.0g
- Sodium lauryl sulphate	0.2g
- Sodium deoxycholate	0.1g
- Bromocresol purple	0.08g
- Bromo phenol red	0.08g
- Agar	15.0g
- dH_2O	

Mix above ingredients and heat to 90°C to dissolve agar. Autoclave for 15 minutes at 121°C / 15 PSI. Cool to 50°C and dispense into sterile square petri dishes. Store at 4°C after solidification. Final pH 7.1 +/- 0.1.

12) - YN17 Blue (Used for the isolation of Bifidobacterium):

- Yeast extract	20.0g
- Polypeptone BBL	10.0g
- Lactose	10.0g
- Casamino acid	8.0g
- Sodium chloride	3.2g
- Bromocresol green0.30g	
- Cysteine hydrochloride	0.40g

- Agar	15.0g
- dH_2O	1000mL

Mix above ingredients on medium heat until agar dissolves. Autoclave for 15 minutes at 121°C / 15 PSI and then cool to 60°C. Stir in *Antibiotic solution. Dispense into sterile petri dishes. After solidification store at 4°C. Final pH 6.9 +/- 0.1.

*Antibiotic solution:

A)	Nalidixic acid	0.03g
B)	Kanaycin sulphate	0.05g
C)	Polymixin B	0.0062g

13) - Growth media for Bifido YN-17

- Follow same procedure as above, however, omit bromocresol green and antibiotic solution

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APPENDIX C

THE ISOLATION AND IDENTIFICATION OF BIFIDOBACTERIA FROM FECAL AND SEWAGE SAMPLES

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LIST OF ABBREVIATIONS

° C = Degree Celsius

FC = Fecal coliforms

G = Grams

hrs = Hours

HBSA = Human bifid sorbitol agar

i.e. = That is

L = Litres

LiCl = Lithium chloride

MF = Membrane filtration

mg = milligrams

mL = Milliliters

min = Minutes

mm = millimeters

m-Tec = Medium for thermotolerant *Escherichia coli*

nm = Nanometres

s = seconds

YN-17 = Medium developed by Mara and Oragui for the enumeration of bifidobacteria

ABSTRACT

The distribution of Bifidobacteria in the environment was studied utilizing two selective media: YN-17 and HBSA. The feces of humans, cats and dogs as well as sanitary and storm sewage samples were analyzed and Bifidobacteria were isolated in all cases except for the dogs. Species of sorbitol-fermenting Bifidobacteria, which have been previously reported as being exclusive to human hosts, were isolated from feces of human and cats as well as from sanitary and storm sewage samples. However, 100% of the isolates from cats did not ferment sorbitol when a carbohydrate profile was carried out. These sorbitol-fermenting species were recovered using both YN-17 and HBSA. However, a higher percentage recovery was made on HBSA as this medium is specific for sorbitol-fermenting species. The results of this study indicate that sorbitol-fermenting Bifidobacteria may be useful as indicators of recent human fecal pollution in surface waters.

INTRODUCTION

Due to the disease hazards associated with fecally-contaminated water, it is imperative that the presence of fecal wastes be rapidly and accurately detected in surface water bodies, and the sources of these inputs traced in order to eliminate them. Thus a reliable bacterial indicator is desired which will detect recent fecal pollution and will in some way differentiate the source of the input (i.e. human and non-human).

In 1981, the Toronto Area Watershed Management Study was initiated to better define the water quality conditions in Humber, Black Creek, Don Rivers and along Toronto Beaches. Particular emphasis was placed on urban areas of Metropolitan Toronto especially on point pollution inputs since high levels of fecal indicating bacteria were found in storm sewages. Subsequent studies began in the fall of 1982 to assess the contribution of pollution loading by the storm sewers and to trace the source of pollution in these lines. Unfortunately, pinpointing the original source of pollution is difficult because a specific bacterial indicator of human fecal wastes is not available at the present time.

Traditionally E. coli and FC have been used as the indicators of fecal pollution in storm waters and surface waters but due to its wide distribution in both human and animal feces they are not acceptable as an indicator of human input only. A number of workers have suggested using Bifidobacterium as a fecal indicator. (Buchanan and Gibbon, 1947; Scardovi et al, 1971; Levin, 1977 and Resnick and Levin, 1981).

The genus Bifidobacterium includes gram-positive, non-sporulating, non-motile, anaerobic, pleomorphic rods. At the present time, there are 24 known species of bifidobacteria that have been identified. Bifidobacteria are present in concentration of 10^9 organisms per gram of feces in humans (Geldriech, 1978), but have a very limited distribution among other animals (Mara and Oragui, 1983). They also have been recovered from raw sewage (Resnick and Levin, 1981) and in a Tropical Rain Forest Watershed B. adolescentis was isolated and enumerated. (Carillo et al, 1985).

The first synthetic selective medium that gave a reasonable recovery of bifidobacteria was invented by Gyllenberg and Niemen in 1960 and was called GN-6. This was modified in 1981 by Resnick and Levin who added nalidixic acid to GN-6 and named it YN-6. Then, in 1983, Mara and Oragui developed YN-17 medium which is more selective for bifidobacteria due to a further modification of YN-6 (i.e. addition of polymyxin B and kanamycin sulphate). These antibiotics decreased the streptococcal contamination in the selective medium but did not eliminate them.

In that same study, Mara and Oragui proposed a methodology to detect bifidobacteria species as indicators of human fecal wastes in surface waters. They devised a medium called HBSA which is very similar to YN-17 except its replacement of fermentive carbohydrate lactose by sorbitol. Their method involved the isolation of bifidobacteria by membrane filtration of test samples onto HBSA which only allowed growth of sorbitol-fermenting species. This study reported that sorbitol-fermenting species of bifidobacteria were exclusive to human fecal wastes. These species were B. adolescentis and B. breve.

OBJECTIVES

The purpose of this project was:

- To test the relative merits of HBSA in conjunction with another standard medium for the isolation and enumeration of bifidobacteria which is YN-17.
- To study the possible usage of MRS medium as a selective medium for bifidobacteria and test the effectivity of LiCl in repression of streptococcal contamination.
- Isolation and speciation of bifidobacteria found in feces of humans, cats and dogs as well as storm and sanitary sewage samples.
- To confirm whether or not sorbitol-fermenting species are found in human waste but are absent in animal wastes.
- To compare the concentration of fecal coliforms and _E. coli_ with bifidobacteria count in feces and in sewage samples.

MATERIALS AND METHODS

Collection and Preparation of the Test Samples

In the fall of 1987 samples were collected from three points along a storm sewage line draining the Danforth-Pape Area and also from two points along a sanitary sewage line located in proximity to the storm water. The samples were collected in triplicates for statistical accuracy. This particular storm sewer line was chosen because it was suspected of having an illegal sanitary hook-up. Water samples were transported to the laboratory on ice in sterile sampling bottles with sodium thiosulphate to neutralize any chlorine that might be present.

Fecal samples were collected in presterilized wide-mouthed jars. A total of 20g of feces was weighed out, diluted 1 in 10 with sterile buffered phosphate water and mixed in a blender for 15s at low speed before being examined. All the samples were analyzed within 2- hours of its collection.

Analysis of Samples

Both the sewage and fecal samples were tested by membrane filtration analysis. This method is one of the standard processes used in water quality monitoring (Dufour and Cabelli, 1975; Dufour et al, 1981). Serially-diluted samples were filtered through Gelman GN-6 47mm nitrate filters having a porosity of 0.45um. A control sample of sterile buffered water was filtered for each of the samples by placing these filters on the least selective medium (i.e. m-Tec). The filters of the samples were planted on the selective media for the recovery of fecal coliforms and bifidobacteria.

FC bacterial densities were determined by using m-Tec agar which was incubated at 44.5 +/- 0.5° C for 23 +/- 1 hrs. The target colonies enumerated were yellow, yellow-green and yellow-brown on this medium. To ensure the accuracy of the counts, filters with 10 - 100 target colonies were chosen to enumerate. These filters were subsequently tested for E. coli confirmation using the urease test. The test involved placing the filter on a pad soaked in a urea phenol red solution and allowed to react for 15 minutes. Deamination by non-E. coli coliform bacteria possessing urease causes a colour change in the colonies from yellow to pink. A second count of urease negative (yellow) colonies was then taken.

The isolation and enumeration of bifidobacteria was carried out using YN-17 and HBSA selective media. These media were incubated anaerobically in jars with hydrogen and carbon dioxide gas packs for 48 hrs at 37° C. The target colonies on the YN-17 medium were characteristically circular, convex, mucoid, 2-3mm in diameter, glistening, greenish-blue and dark blue and on HBSA they were brownish-yellow to yellow. Streptococcal contamination on YN-17 was exhibited by the presence of the greenish-blue colonies with pale green periphery an on HBSA by light yellow and clear colonies. These colonies were not enumerated and were considered as background. For the formulation of the media used see Appendix.

Identification of Isolates

From the total count of bifidobacteria that exhibited the typical target morphology on the selective media, 10% were isolated to be identified. These isolates were streaked out on a growth medium for purity. The growth medium used was YN-17 base without the antibiotics and the indicator. After incubation these isolates were Gram stained and those rods with typical pleomorphic morphology (i.e. bifurcated v an Y forms, club shaped ...) were streaked out for the second time on the growth medium for purity and incubated both anaerobically and aerobically. Isolates exhibiting either gram-negative rods or gram-positive cocci morphology were discarded. Only isolates which grew anaerobically were Gram stained once again and those with typical pleomorphism were considered as bifidobacteria.

The confirmation of bifidobacteria was further verified by carrying out the following biochemical tests: catalase, gelatin and arginine, Kliger's iron and bile esculin. Then the speciation of the bifidobacteria isolates was accomplished by observing the fermentative reactions of the isolates in nine different sugars. The typical fermentation patterns of Bifidobacterium species is illustrated in Table 1.

Carbohydrate Fermentation Profile

The carbohydrate media was made by dissolving 8.0g of phenol red broth base in 450 mL distilled water. A filter sterilized sugar solution was added to this autoclaved broth to give final concentration of 1%. The sugars used were the following: L(+) Arabinose, D-Cellobiose, D-Mannitol, D(+)-Melezitose, D-Ribose, D-Sorbitol, soluble starch and D(+)Xylose. The test tubes of carbohydrates were incubated for 24 hrs at 37° C before the inoculation of the isolates in order to reduce oxygen tension and to ensure sterility. The inoculated tubes were read after anaerobic incubation of 48 hrs and if the results were not definite, they were reincubated for an additional 24 hrs.

Table C-1

Table 1. The carbohydrate fermentation reaction profile used to speciate the Bifidobacteria isolated from the fecal and sewage samples. (formulated from the Bergeys Manual)

Bifidobact. species	Arabnose	Cellobiose	Lactose	Mannitol	Melezitose	Ribose	Sorbitol	Starch	Xylose
bifidum	-	-	+	-	-	-	-	-	-
longum	+	-	+	-	+	+	-	-	v
infantis	-	-	+	-	-	+	-	-	v
breve	-	v	+	v	v	+	v	-	-
adolescentis	+	+	+	v	+	+	v	+	+
angulatum	+	-	+	-	-	+	v	+	+
catenulatum	+	+	+	v	-	+	+	-	+
pseudocatnulatum	+	v	+	-	-	+	v	+	+
dentium	+	+	+	+	+	+	-	+	+
globosum	v	-	+	-	-	+	-	+	v
pseudolongum	+	v	v	-	v	+	-	+	+
cuniculi	+	-	-	-	-	-	-	+	+
choerinum	-	-	+	-	-	-	-	+	-
animalis	+	v	+	-	v	+	-	+	+
thermophilum	-	v	v	-	v	-	-	+	-
boum	-	-	v	-	-	-	-	+	-
magnum	+	-	+	-	-	+	-	-	+
pullorum	+	-	-	-	-	+	-	-	+
suis	+	-	+	-	-	-	-	-	+
minimum	-	-	-	-	-	-	-	+	-
subtile	-	-	-	-	+	+	+	+	-
coryneforme	+	+	-	-	-	+	-	-	-
asteriodes	+	+	-	-	-	+	-	-	+
indicum	-	+	-	-	-	+	-	-	-

"+"= The sugar was fermented

"-"= The sugar was not fermented

"v"= Some strains ferment this sugar

Flash Freezing

The ATCC cultures were flash frozen for long-term storage. The cultures were prepared by growing on a growth medium for purity and quantity. Then under the Biohazard Hood, approximately 5 mL of 40% glycerol was poured onto each of the plates and with a flamed loop the colonies were dispersed. By using 1 mL pipettes the culture-glycerol solutions were soaked into small pieces of filter papers which had been aseptically placed in labelled freezer bags. These were done in triplicates and were heat-sealed. The bags were placed in a small box and stored in a -70° C freezer.

The frozen cultures were revived by planting the saturated filters on the growth medium and streaking for isolated colonies.

Results

The YN-17 medium allowed more accurate enumeration and isolation of bifidobacteria than HBSA as indicated by the higher percentage of false positive targets (i.e. 10% for HBSA and 5% for YN-17). A large majority of these false positive targets were fecal streptococci. MRS agar with the antibiotic cocktail of YN-17 was also tested for its selectivity of bifidobacteria. Unfortunately, this medium supported growth of both streptococci and Lactobacillus species which resulted in higher concentrations of background bacteria. However, this medium shortened the incubation period by half and thus was used without the antibiotics as a growth medium. In addition, LiCl was incorporated at the concentrations of 0.3%-0.4% to YN-17 and HBSA; however it did not decrease the streptococcal contamination as was suggested by N.A. Klapes at the ASM meeting in March of 1987.

The geometric mean concentrations of FC, E. coli and bifidobacteria in storm and sanitary sewage samples can be seen in Table 2. Also, a representative graph of the counts from storm sewage samples is illustrated in Figure 1. Both Table 2 and Figure 1 indicate that in storm sewers total bifidobacteria exist in higher concentrations than FC or E. coli. The table and figure also show the trend towards decreasing concentrations of all the bacteria from points A to C due to dilution and die-off within the storm sewer line. From the graph one can clearly see that in storm sewage bifidobacteria exist in concentrations about 10 times greater than E. coli . Note that there are higher concentrations of bacteria in sanitary sewage than in storm sewage and that variance in bacterial counts occurs over the different sample days.

Results of the fecal analysis (Table 3) also illustrates a higher concentration of bifidobacteria than E. coli (i.e. 10 - 100 times greater). Bifidobacteria were isolated from the feces of humans and cats but not from dogs. The sewage samples had lower concentrations of bifidobacteria and E. coli than the fecal samples. This is due to dilution and to the fact that the organisms die off at a faster rate in the environment (Oragui, 1982).

The fermentative reaction of ATCC cultures of bifidobacteria (Table 4) closely corresponds with those of the Bergey's Manual profile used for speciation of the isolates (Table 1). Table 5 illustrates the biochemical reactions used to confirm bifidobacteria (Bergey's Manual 9th ed.). The ATCC cultures exhibited typical biochemical reactions for this genus (i.e. catalase (-), arginine (-), acid production in Kliger's iron medium and variable hydrolysis of bile esculin).

Recovery of bifidobacteria species from feces and sewage on two selective media is shown in Table 6 and Figure 2. A wider variety of bifidobacteria

Table C-2

Table 2. Geometric mean concentrations of Fecal Coliforms. Escherichia coli and Bifidobacteria in the storm and sanitary sewage samples.

Date (1986)	sample & its source	FC	EC	HBSA	YN-17
		(counts per 100 ml of sample)			
	storm sewage				
Oct 22	A	3933	3617	-	11466
Oct 28	A	10233	10200	133000	270000
Nov 18	A	59666	57500	21000	460000
	B	26000	23700	1600	52000
	C	1300	830	1500	4800
	sanitary sewage				
Oct 28	D	253000	220000	-	96000
Nov 18	D	2366000	1700000	67000	190000
	E	3730000	2600000	47000	60000
Jan 31	E	-	2900000	42000	57000

YN-17 = Total count of Bifidobacteria

HBSA = Count of the sorbitol fermenting Bifidobacteria

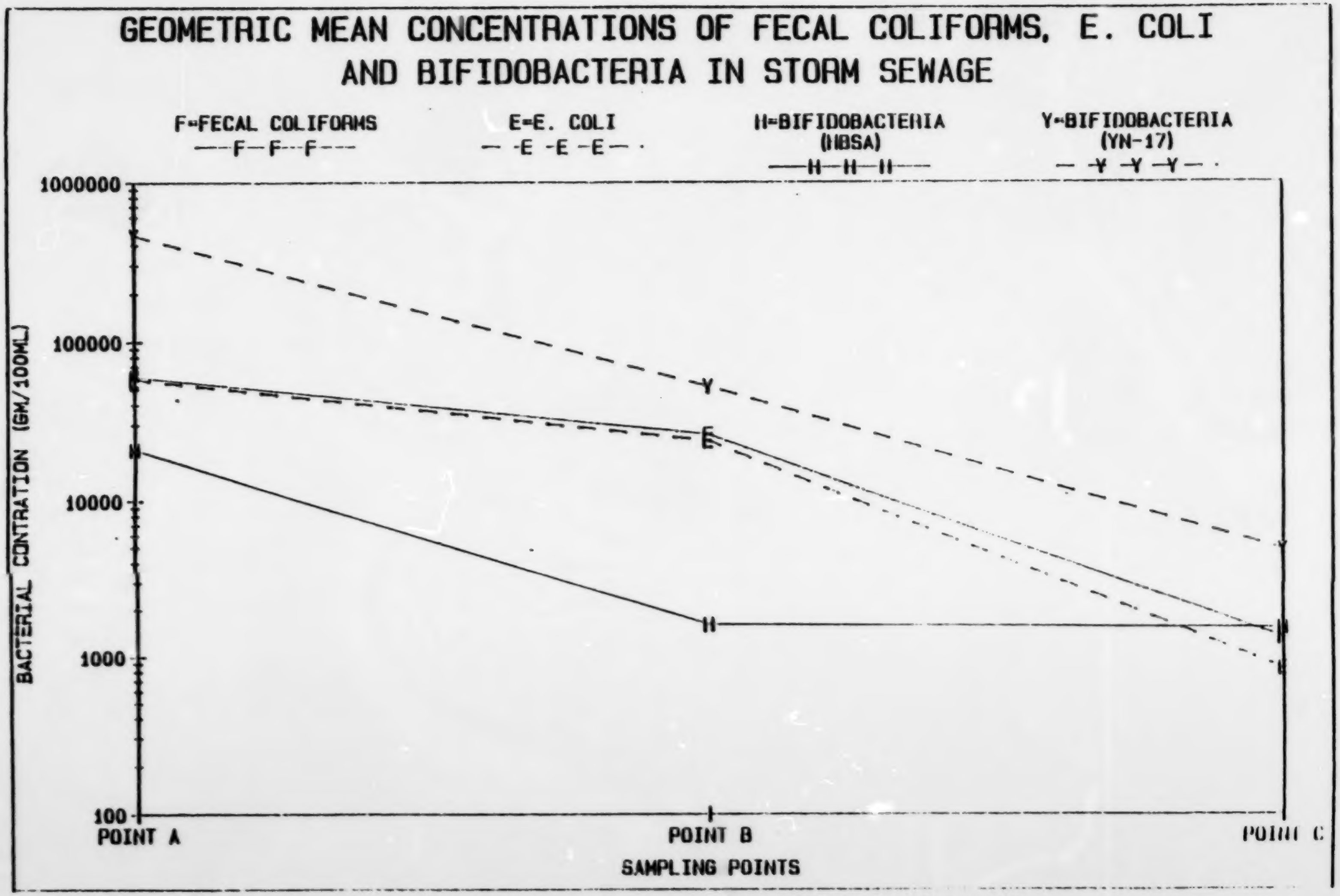

Figure C-1 GEOMETRIC MEAN CONCENTRATIONS OF FECAL INDICATING BACTERIA IN STORM SEWAGE.

Table C-3

Table 3. Geometric mean concentrations of Escherichia coli and Bifidobacteria in the feces of humans, cats and dogs.

Source	EC	HBSA	VN-17
		(per 1 gram of feces)	
human	230,000,000	20,000,000	37,000,000,000
cat	6,400,000	17,000,000	526,000,000
dog	8,067,000	0	0

Table C-4

Table 4.The characteristic fermentative reactions of 9 sugars by the ATCC cultures of Bifidobacteria.

Species	arabnose	cellobiose	lactose	mannitol	melezitose	ribose	sorbitol	starch	xylose
bifidum	-	-	+	-	-	-	-	-	-
infantis	-	-	+	-	-	+	-	-	-
parvulorum	+	-	+	-	+	+	-	+	-
breve	-	+	+	-	-	+	+	-	-
liberorum	-	-	+	-	-	+	-	-	-
adolescentis	+	+	+	-	+	+	-	+	+
longum	+	-	+	-	+	+	-	-	-

Table C-5

Table 5. The biochemical test scheme used for the identification of Bifidobacteria in the profiles of ATCC cultures and their Gram Stain Characteristics.

ATTC #	Bifidobact. species	morphology	Arginine hydrolysis	B.esculin hydro.	Gelatin hydro.	Kliger's iron	catalase
15696	bifidum	G +ve pleomorphic "amphora-like" cells	-	-	-	A	-
15697	infantis	G +ve pleomorphic	-	+	-	A	-
15698	parvulorum	G +ve branching, long, thin rods	-	-	-	A	-
15700 15701	breve	G +ve thin & short rods	-	+	-	A	-
15702	liberorum	G +ve long & short thin rods	-	-	-	A	-
15703 15704	adolescentis	G +ve pleomorphic having many of other species' morphology	-	+	-	A	-
15707	longum	G +ve long cells with club shaped ends	-	-	-	A	-

"A" = Acid production
"+" = Hydrolysis was observed
"-" = Hydrolysis was not observed

Table C-6

Table 5. Species of Bifidobacteria isolated from the two selective media that occurs in fecal and sewage samples.

Bifidobacterium species	Source	HBSA	YN-17
adolescentis	human	6/9	8/16
	cat	-	-
	storm sewage	-	4/4
	sanitary sewage	11/18	10/19
longum	human	1/9	7/16
	cat	-	-
	storm	-	-
	sanitary	-	-
breve	human	2/9	1/16
	cat	12/18	-
	storm	-	-
	sanitary	-	3/19
globosum	human	-	-
	cat	-	-
	storm	-	-
	sanitary	1/18	1/19
pseudocatnulatum	human	-	-
	cat	1/18	-
	storm	1/1	-
	sanitary	-	-
suis	human	-	-
	cat	-	-
	storm	-	-
	sanitary	-	1/19
angulatum	human	-	-
	cat	1/18	-
	storm	-	-
	sanitary	3/18	2/19
thermophilum	human	-	-
	cat	1/18	-
	storm	-	-
	sanitary	-	-
chorium	human	-	-
	cat	-	-
	storm	-	-
	sanitary	-	-
unidentifiable	human	-	-
	cat	3/18	-
	storm	-	-
	sanitary	2/18	2/19

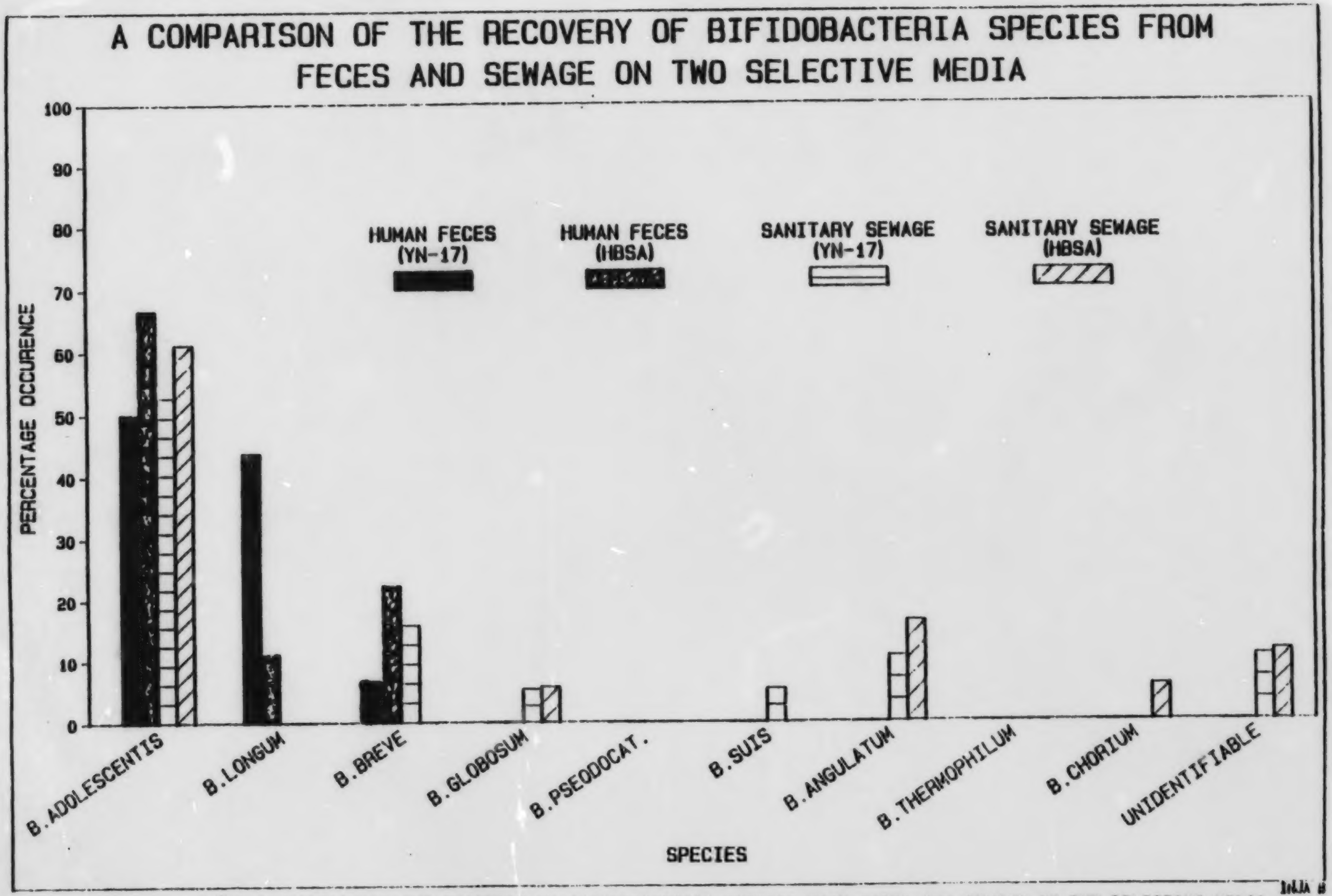

Figure C-2 A COMPARISON OF THE RECOVERY OF BIFIDOBACTERIA SPECIES FROM FECES AND SEWAGE ON TWO SELECTIVE MEDIA.

species were recovered in the sewage samples than in fecal samples (Fig. 3). Figure 3 illustrates that the predominant species in human feces and sewage were B. adolescentis and B. breve which are the sorbitol-fermenting species of bifidobacteria (Mara and Oragui, 1983). As indicated by Fig. 3 B. breve was isolated from cat samples but 100% of these isolates did not ferment sorbitol when the carbohydrate profile was carried out.

Discussion

The superiority of the two selective media is due to the antibiotic, kanamycin sulphate, which reduced levels of the contaminating streptococci greatly when used in the specified concentration. However, it didn't completely eliminate the background of streptococci. Yield of bifidobacteria was higher on YN-17 than HBSA because HBSA was a more selective medium in that it only allowed growth of sorbitol-fermenting species. In addition, target colonies were more easily distinguished on YN-17, while there was a tendency towards more false (+) targets arising from streptococci on HBSA.

The data presented herein disagreed with the study done by Mara and Oragui in 1983. In this project it was discovered that their description of Bifidobacterium on YN-17 (i.e. blue centre with pale green periphery) was in fact a mixture of bifidobacteria and streptococci. Thus, during the beginning of the year the successful recovery rate of bifidobacteria was considerably lower; however, accuracy of 95% and higher was achieved during the latter part of the year.

Bifidobacteria can be used as fecal indicating bacteria because they exist in high number in both feces and fecally contaminated environments. They also

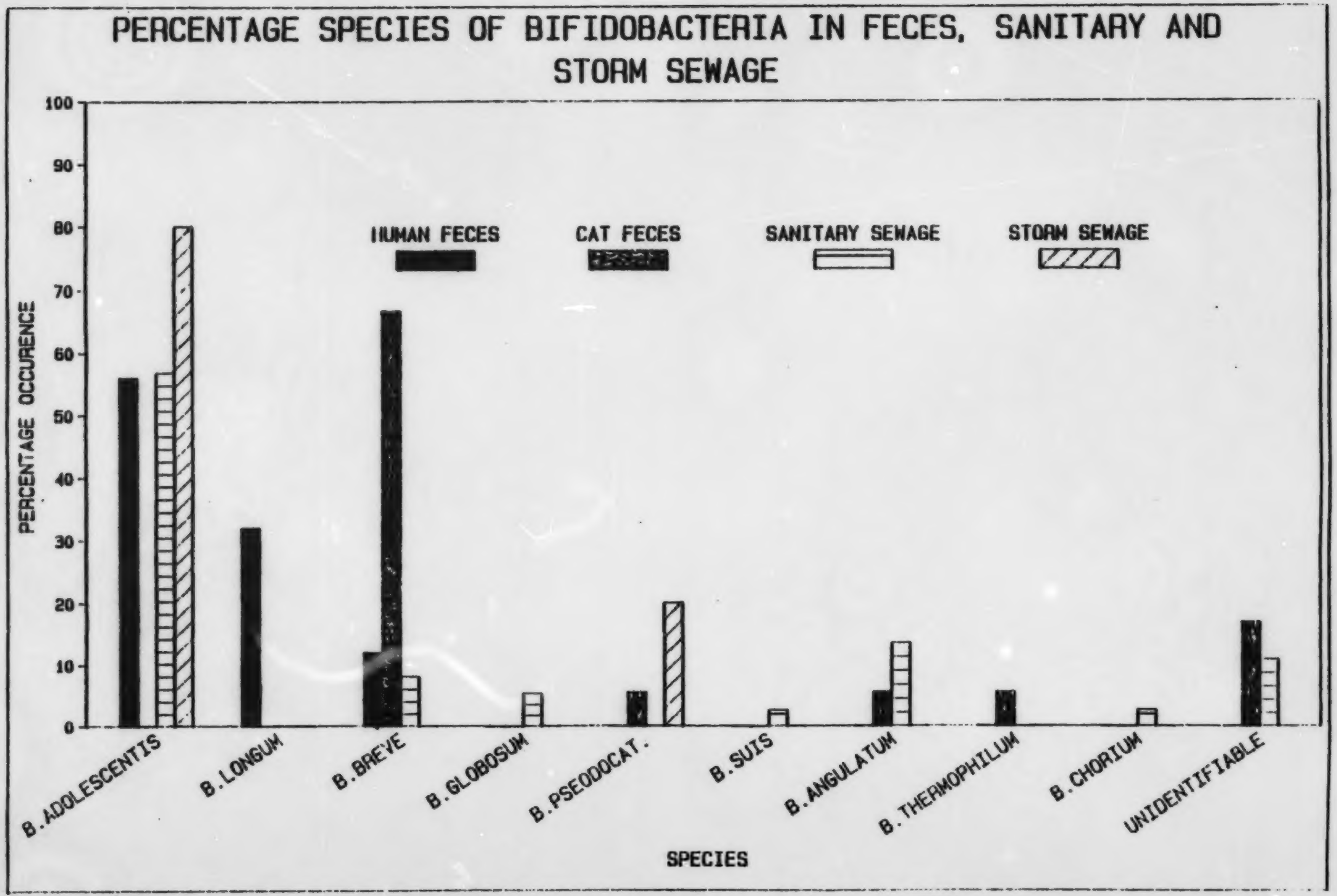

Figure C-3 PERCENTAGE SPECIES OF BIFIDOBACTERIA IN FECES, SANITARY AND STORM SEWAGE.

do not multiply outside the body and a rapid methodology for recovery from the aquatic environment is available as illustrated in this study.

Site A of the storm sewer line is the likely location of the illegal connection since the concentration of fecal indicating bacteria was highest at this in-line point. The illegal hook-up is also confirmed by the presence of sorbitol-fermenting bifidobacteria in the line. Sanitary sewage samples contained a wide variety of species of Bifidobacterium which is possibly due to some inputs of animal wastes as well as humans, but the highest recoveries made were again of sorbitol-fermenting bifidobacteria and this correlates with the face that human feces make up the greatest percentage of wastes in sanitary sewage. Although bifidobacteria can be recovered from cats, it is unlikely that cat feces represent a major source of fecal contamination in the storm sewer line. Therefore sorbitol-fermenting species can be used to monitor recent human fecal contamination in sewage lines.

Conclusions

- YN-17 yielded higher concentrations and represented a broader spectrum of different species of bifidobacteria than HBSA.
- B. adolescentis was the most prevalent species of bifidobacteria in human feces and sewage samples, but were absent in cats.
- Sorbitol-fermenting species (i.e. B. breve) were isolated from both human and cat feces but 100% of the isolates from the cats didn't ferment sorbitol when the carbohydrate fermentation profile was carried out.
- Bifidobacteria counts were 100 times greater than E. coli in feces and 10 times greater in sewage samples.

Recommendations

- Further study to confirm the specificity of the selective media for bifidobacteria.
- Carry out a large scale fecal sample study to see if the sorbitol-fermenting species, B. adolescentis, is predominant in human feces and is limited or absent in animal feces.

Acknowledgements

The author would like to sincerely thank Elizabeth Harris and Dr. P.L. Seyfried for their support throughout the year.

Appendix

1. Dissolve 34.0g of KH_2PO_4 in 500 mL distilled water and adjust the pH to 7.2. Then dilute this to 1L by adding distilled water.
2. Dissolve 50 g of $MGSO_4$ $7H_2O$ in 1L of distilled water.

These two solutions were autoclaved separately for 15 min at 121° C. After these were cooled to room temperature, add 1.25 mL of 1) and 5 mL of 2) to 1L of distilled water. Then it can be dispensed either as dilution blanks or as rinse water for M.F. and autoclave again at 121° C for 20 minutes.

m-Tec Agar

This medium is used to isolate and enumerate the Fecal Coliforms as well as the Thermo-tolerant E. coli.

Proteose peptone #3	10.00 g
Yeast extract	3.00 g
Lactose	10.00 g
NaCl	7.50 g
K_2HPO_4	3.30 g
KH_2PO_4	1.00 g
Sodium lauryl sulphate	0.20 g
Sodium deoxycholate	0.10 g
Bromocresol purple	0.08 g
Bromophenol red	0.08 g
Agar	15.00 g
Distilled water	1.00 L

The above ingredients were mixed and heated to dissolve. Autoclaved for 15 min at 121° C and dispensed into Petri dishes after it is cooled. Before pouring the pH is adjusted to 7.1 +/- 0.1.

The two selective media for Bifidobacteria

	YN-17	HBSA
Yeast extract	20.00 g	20.00 g
Poly-peptone (BBL)	10.00 g	10.00 g
Lactose	10.00 g	10.00 g
Casamino acid	8.00 g	8.00 g
NaCl	3.20 g	3.20 g
Bromocresol green	0.30 g	-
Bromocresol purple	-	0.10 g
Cystein hydrochloride	0.40 g	0.40 g
Agar	15.00 g	15.00 g
Distilled water	1.00 L	1.00 L

The above ingredients were mixed in a 6L beaker and were stirred on medium heat until agar was dissolved. Then it was autoclaved for 15 minutes at 121° C. After the isolation was cooled to 60° C the following were added:

Nalidixic acid	30.0 mg	20.0 mg
Kanamycin sulphate	50.0 mg	50.0 mg
Polymixin B	6.20 mg	1.20 mg

Finally, the pH was adjusted to 6.9 +/- 0.1 and then the media were poured into square Petri dishes (100 cm^2).

Distinct Gram Morphologies that are Species Characteristics of Bifidobacteria

Bifidobacterium species	Gram morphology
bifidum	- highly variable but has the typical "amphora-like" shaped cells
longum	- very elongated and relatively thin cell with slightly irregular contours and rare branching
breve	- thinnest and shortest rods
angulatum	- v(angular) or palisade arrangement similar to those of corynebacteria
catenulatum	- chains of 3, 4 or more globular elements - distinct branching
globosum	- short, coccoid or almost spherical to curved or tapered - arranged in singly or doubly or rarely in short chains
animalis	- characteristic central portion slightly enlarged

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APPENDIX D

A STUDY OF THE SURVIVAL OF BIFIDOBACTERIA AND THEIR ROLE IN WATER QUALITY CONTROL

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
dH_2O	distilled water
g	grams
h	hours
HBSA	Human Bifid Sorbitol Agar
min	minutes
mL	milliliters
m-TEC	Medium for selection of thermotolerant E. coli
MPN	Modification of Petuely's PMS for the isolation of bifidobacteria
MRS	Medium for the selection of lactobacillia used also for isolation of bifidobacteria
YN-17	Medium for the isolation of bifidobacteria
YN-17(-)	Non-selective medium used to support the growth of bifidobacteria (Modification of YN-17)

INTRODUCTION

The relatively recent closure of a number of Toronto beaches due to fecal pollution signifies a need to improve water quality in this area. Outbreaks of various infections traced to fecal contamination of surface waters are well-documented (Dart and Stretton, 1980).

Development of a management strategy plan to reduce fecal contamination requires suitable detection methods that can locate fecal inputs and differentiate between human vs. non-human sources of pollution.

Ideally the enumeration of pathogen concentrations in surface waters would serve to indicate levels of contamination and potential health hazards. However, pathogens pose problems due to their sporadic occurrence in the environment and the difficulty in their collection and isolation. Hence, a more suitable surrogate indicator organism found in feces must be employed.

Geldreich, in 1968, and Bonde, in 1983, have proposed a number of parameters for an ideal water quality indicator. Briefly stated: i) the indicator should exist at density levels far exceeding pathogen concentrations in feces of infected individuals; ii) the indicator should only be present in warm-blooded animals; iii) there should exist a relatively rapid and inexpensive means of detecting and enumerating the indicator; iv) indicator's survival characteristics should parallel those of the pathogen(s).

The most widely accepted indicator of water quality impairment due to human and animal excrement is the fecal coliform count. The Ontario Ministry of the Environment utilizes a standard of 100 fecal coliforms/100 mL of water as their upper limit of safety (MacDonald, 1986). However, this indicator has a drawback in that it enumerates Klebsiella which is not restricted to feces (MacDonald, 1986).

Escherichia coli is also being utilized as an indicator of fecal contamination in surface waters. Unlike other fecal coliforms it has not been isolated from non-fecal sources. Due to its fairly rapid die-off *in situ*, *E. coli* has been employed as an indicator of recent fecal contamination (Seyfried *et al*., unpublished report).

Unfortunately, the fecal coliforms, including *E. coli*, when used as water quality indicators, are incapable of distinguishing between human and animal sources. These organisms are ubiquitously found in higher animals.

Mossel (1958) was the first to propose bifidobacteria as potential indicators of fecal contamination. *Bifidobacterium* is a gram-positive, anaerobic, non-sporulating, non-motile rod often displaying pleomorphic forms (i.e. Y and V-shaped bifurcated rods).

Several media have been proposed for the isolation of bifidobacteria. In 1983, Mara and Oragui developed YN-17 medium stating relatively good selectivity for bifidobacteria. Tanaka and Mutai (1980) stated similar results with the use of MPN medium, a modification of Petuely's Synthetic Medium (PSM) (Petuely, 1956). MRS, a medium normally utilized for the isolation of *Lactobacillus spp.* has been employed for the isolation of bifidobacteria by the Department of Nutrition at the University of Toronto (personal communication). Most contamination reported on these three media was the result of fecal streptococci growth. Lithium chloride was found to control unwanted growth of *Streptococcus spp.*. It was found to exert an inhibitory effect at concentrations of 0.3% and 0.4% in MSLSM *Staphylococcus aureus* medium (N.A. Klapes, Annual Meeting, American Society of Microbiologists 1987). Its effectiveness in selective media for bifidobacteria has yet to be investigated.

It has been suggested that bifidobacteria could be used to distinguish between human and animal fecal pollution (Buchanan and Gibbons, 1974; Levin, 1977; Cabelli, 1979; Resnick and Levin, 1981b). However, it was not until 1983 that a method was devised by Mara and Oragui that would allow bifidobacteria to serve this purpose. Using a sorbitol-based medium (HBSA), they isolated and analyzed bifidobacteria from human and animal feces. Their findings showed that certain sorbitol-fermenting species were restricted to human feces.

The usefulness of bifidobacteria as a specific indicator of human fecal pollution could be limited due to its short survival in the extra-intestinal environment (Resnick and Levin, 1981; Oragui, 1982). Survival studies have shown bifidobacteria to die off more rapidly than _E. coli_ upon exposure to environmental waters (Carillo, Estrada and Hazen, 1985). A too rapid decline of bifidobacteria in the aquatic environment and subsequent inability to detect their presence could render the organisms poor indicators of fecal contamination.

In 1972, McFeters and Stuart devised dialysis membrane filter chambers for _in situ_ and _in vitro_ survival studies. The chamber allows for maximum exchange of molecules (e.g. gases, organic nutrients, toxic chemicals) between the environment and a pure culture of bacteria without contaminating the culture by introducing new water-borne bacteria into it.

Before bifidobacteria can be used as an indicator of recent human fecal contamination in the Toronto area, their survival in this environment and their ability to satisfy the parameters of a good indicator must be investigated.

OBJECTIVES OF RESEARCH

1. To assess the suitability of three media: YN-17, MPN, and MRS; in their selectivity for bifidobacteria.
2. To enumerate bifidobacteria (all species), bifidobacteria (sorbitol-fermenting) and *E. coli* from human feces.
3. To test the inhibitory effects of lithium chloride on bifidobacteria and fecal streptococci utilizing YN-17.
4. To compare the survival characteristics, *in vitro*, of mixed cultures and laboratory isolates of bifidobacteria with those of *E. coli*.
5. To investigate the suitability of bifidobacteria as indicators of recent human fecal pollution.

METHODS AND MATERIALS

Part 1: Isolation and Enumeration

SAMPLE COLLECTION

Feces were collected from healthy individuals, in sterile, wide-mouthed glass jars. All samples were freshly voided and usually examined in the laboratory within four hours of defecation.

SAMPLE PREPARATION

A total of 20g of each fecal sample was weighted out and transferred to sterile glass blender jars which had been calibrated previously to a 200mL volume. Sterile phosphate solution (Appendix 1) was added to the 200mL volume mark producing a 10^{-1} dilution of the original sample. The diluted samples were homogenized for 15 seconds at low speed using a Waring blender. Systematic 100-fold dilutions were made of the blended samples in sterile 99mL dilution blanks (Appendix 1) prior to membrane filtering.

Membrane Filtration

Dilutions of the fecal samples were membrane filtered according to the methods outlined in Standard Methods for the Examination of Water and Wastewater (1980). The filtrations were performed utilizing Gelman GN6 47mm cellulose nitrate filters with a porosity of 0.45um. Filters were planted on the appropriate selective media. For each sample a control was run on the least selective medium which was usually m-TEC.

SELECTIVE MEDIA

m-TEC medium (Dufour, 1975) and the Urease Test (Dufour, 1981) were employed for the selection and enumeration of *E. coli* from the samples tested. The medium was incubated aerobically for 24h at 44.5° C in the presence of two plastic containers, holding 50mL of ice each, to ensure a resuscitation step necessary for target colony growth. The m-TEC medium (Appendix 2) is selective for all fecal coliforms present in the samples. Target colonies of all yellow, yellow-green and yellow-brown colonies were enumerated as the fecal coliform count. To differentiate E. coli from other thermotolerant fecal coliform bacteria, the Urease Test was employed. The sample filter was removed from m-TEC and placed onto a sterile filter pad saturated with a urease solution (Appendix 1). All urease-negative (yellow) colonies were recorded as the E. coli count.

The MPN medium formulated by Tanaka and Mutai (1980) was utilized to select for bifidobacteria. MPN medium (Appendix 2) was incubated anaerobically at 37^{o} C for 48h with GasPaks (BBL) in an atmosphere of 95% hydrogen and 5% carbon dioxide. Target colonies were round, 1-2mm in diameter, convex, flat with an entire edge and creamy whitish-yellow in appearance.

MRS medium (Appendix 2) was proposed as being selective and specific for bifidobacteria (Department of Nutrition, University of Toronto, 1987). The medium was incubated anaerobically at 37^{o} C for 24h with GasPaks (BBL) in an atmosphere of 95% hydrogen and 5% carbon dioxide. Presumptively positive bifidobacteria colonies were those that were circular, 2-3mm in diameter, dark green or blue, convex, and mucoid. Fecal streptococci contamination was observed as the presence of colonies 1-2mm in diameter round and green with a pale green periphery.

The YN-17 medium (Appendix 2) formulated by Mara and Oragui (1983) was also utilized for its selectivity and specificity for bifidobacteria. The medium was incubated anaerobically for 48h employing the same anaerobic techniques used for MPN and MRS media. Target colonies and background contamination were identified by the same criteria used for the MRS medium.

Human Bifid Sorbitol Agar (HBSA) (Appendix 2) was formulated by Mara and Oragui (1983). The medium was used to select for sorbitol-fermenting bifidobacteria. Incubation procedures were consistent with those used for YN-17 medium. Target colonies were taken as those colonies that were deep yellow and brownish-yellow, 2-3mm in diameter, circular and convex. Contamination of fecal streptococci were those colonies measuring 1-2mm in diameter, flat, pale yellow or colorless.

Concentrations of 3% and 4% LiCl were incorporated into a separate batch of YN-17 media to test for suppression of fecal streptococcal growth. Incubation procedures and target and non-target identification criteria were consistent with those utilized for the YN-17 medium.

ENUMERATION OF BIFIDOBACTERIA AND E. COLI

Human fecal samples were obtained and processed as mentioned above. Enumeration of bifidobacteria and E. coli was performed using the membrane filtration method and the two media, m-TEC and YN-17.

IDENTIFICATION OF BIFIDOBACTERIA

Colonies from YN-17, MPN, and MRS were picked and streaked on YN-17(-) (Appendix 2) to purify. Isolates were classified as gram-positive or gram-negative organisms. Gram-positive rods exhibiting the typical pleomorphic morphology were restreaked and incubated in aerobic and anaerobic environments. Those isolates growing anaerobically were presumed to be bifidobacteria and were subjected to further biochemical tests. The confirmation was performed by Inja Huh (1986-87) using the following biochemical tests: catalase, gelatin, arginine, bile esculin, and Kliger's iron. Further identification of the isolates to the species levels was accomplished using carbohydrate fermentation tests (Huh, 1986-87).

Part II: Survival Study

ASSEMBLY AND USE OF DIFFUSION CHAMBERS

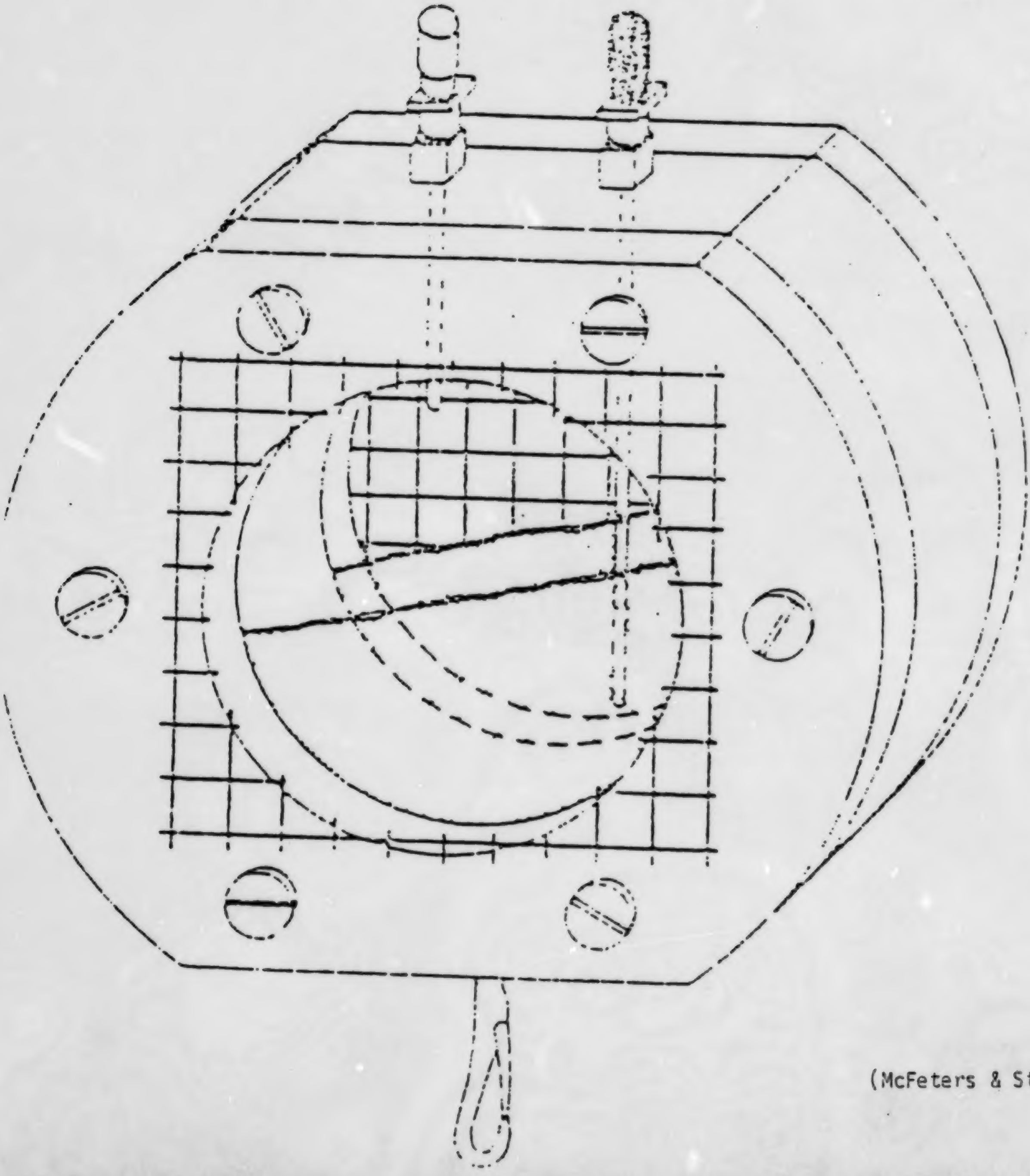
50mL dialysis membrane diffusion chambers used in the survival study were designed by McFeters and Stuart (1972). The chamber consists of a three-part plexiglass II cell with membrane filter sidewalls (Fig. 1). Filter sidewalls were made of a polycarbonate film with a 99mm diameter and a porosity of 0.2um (Nucleopore). Filters were placed between the plexiglass outer walls and the centre piece. Wing nutbolts were loosely set in place to hold the assembled chambers together. The chambers were autoclaved for 25min. at 121° C (15lbs pressure). Before use, the wing nutbolts were tightened.

PREPARATION OF PURE BACTERIAL CULTURES FOR USE IN DIFFUSION CHAMBERS

Pure cultures of bifidobacteria and E. coli were prepared by inoculating 5mL of MRS broth (Appendix 2) with a single colony from a pure culture of bacterium grown overnight on YN-17(-) (Appendix 2). The broth cultures were incubated for 48h at 37° C. Broth cultures were subjected to centrifugation at 300 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 5mL of chilled gelatin phosphate buffer (Appendix 1) and recentrifuged. This step was repeated twice. After the supernatant was discarded, the cells were resuspended once again in 5mL of sterile gelatin phosphate buffer and measured spectrophotometrically at 660nm. For bifidobacteria an optical density reading of greater than 1.0 signified a culture content of approximately 10^7 bacterial cells. For E. coli, an optical density reading of greater than 1.1 signified a culture content of approximately 10^8 bacterial cells. Further preparation of the cultures was performed as follows:

Figure D-1

DIALYSIS DIFFUSION CHAMBER



(McFeters & Stuart, 1972

i) Tenfold dilutions of the culture were made up to a 1 in 10^8 dilution of the original culture in 99mL dilution blanks (Appendix 1). The dilutions were analyzed by membrane filtration and the filters placed onto appropriate selective media (i.e. m-TEC for E. coli and YN-17 for bifidobacteria). The media were incubated according to the medium and genus requirements, as earlier stated, and subsequent colony counts were obtained to calculate the cell density of the original pure culture.

ii) A 1 in 10^4mL dilution was used to fill the chambers so that the approximate cell density would fall between 10^4 and 10^5 organisms per mL. This dilution was achieved by transferring 3mL of the 1 in 10^2mL dilution to a flask containing 297mL of sterilized environmental water. Table 1 presents a list of the locations from which the environmental water was obtained for each culture utilized in the survival study. The sterile, assembled chambers were inoculated with the pure bacterial culture using a sterile 50mL luer-lock syringe. The chambers could accommodate slightly more than the specified volume and were therefore filled to capacity. The chambers were then placed in a glass tank containing environmental water at room temperature.

PREPARATION OF FECES FOR USE IN DIFFUSION CHAMBERS

A human fecal sample was obtained and prepared as outlined in Part I, Materials and Methods, of this report. The sample was diluted to a 1 in 10^5mL dilution and 3mL of this dilution were placed in 297mL of sterilized environmental water. Chambers were filled as stated above.

Table 1

SOURCE OF ENVIRONMENTAL WATER USED IN VITRO
FOR THE STUDY OF BIFIDOBACTERIAL SURVIVAL

IDENTIFICATION CODE	TYPE OF ISOLATE	ENVIRONMENTAL WATER LOCATION
308	fecal isolate	Lake Ontario: Harbourfront Queen City Yacht Club dock
701	B. breve	Lake Ontario: at foot of Brimley Road
696	B. bifidum	
P-Z	feces	Lake Ontario: Kew Beach near Woodbine Race Track
EY-6	sewage isolate	

SAMPLING OF CHAMBERS

The chambers were sampled at 2, 5, 12, 24, 48, and 72 hours using 10mL disposable luer-lock syringes. The syringe was locked onto the port possessing the attached capillary tube and pumped five times to resuspend settled cells prior to removing the sample. Volumes from 1 to 12mL were removed, appropriately diluted, membrane filtered, and planted on both YN-17 and m-TEC. Bacterial densities were enumerated after incubation.

NOTE: All samples for the survival study were performed in triplicate.

RESULTS

Part 1: Isolation and Enumeration

Of the three media tested for their selectivity and specificity for bifidobacteria from human feces, MRS displayed a high density of contamination. Colonies obtained included streptococci, and lactobaccili as well as bifidobacteria. Hence, the medium was found to be non-specific for bifidobacteria.

YN-17 and MPN were found to be equally selective for bifidobacteria with less than 3% contamination by fecal streptococci. Table 2 shows that the two media were equal in their ability to recover bifidobacteria from the fecal samples.

MPN required an incubation time of 48h before visible growth occurred, exceeding the incubation time required for YN-17 by 24h.

Results of enumeration of bifidobacteria on YN-17 and E. coli on m-TEC are presented in Table 3. Bifidobacterial counts from human feces were found to be greater than counts of E. coli by a factor of approximately 10^3. Table 4 presents results obtained using HBSA. Sorbitol-fermenting bifidobacteria were found in equal densities to those of E. coli. YN-17 medium supplemented with LiCl concentrations of 3% and 4% displayed fecal streptococci contamination accounting for < 8% of the total colonies isolated.

Part 2: Survival of Bifidobacteria and E. coli (Table 5)

Pure Culture of Bifidobacteria Isolated from Sewage

The die-off patterns of bifidobacteria and E. coli appeared to parallel each other (Fig. 2). However, bifidobacteria could not be recovered after the

TABLE D2

COMPARISON OF LEVELS OF BIFIDOBACTERIA ISOLATED FROM HUMAN FECES ON YN-17 AND MPN

SAMPLE SOURCE	**Bifidobacteria Per 1 Gram of Human Feces**	
	YN-17	**MPN**
Human I	2.1×10^9	2.0×10^9
Human A	1.8×10^9	2.0×10^9
Mean	2.0×10^9	2.0×10^9

TABLE D3

LEVELS OF BIFIDOBACTERIA AND ESCHERICHIA COLI IN HUMAN FECES AS ISOLATED ON YN-17 AND M-TEC

SAMPLE SOURCE	**Microorganisms Per Gram of Feces**	
	Bifidobacterium spp[a]	Escherichia coli[b]
Human A	2.3×10^{9}	1.8×10^{7}
Human B	2.6×10^{9}	2.0×10^{7}
Human C	1.1×10^{9}	1.6×10^{7}
Human D	1.2×10^{10}	9.4×10^{6}
Human E	7.9×10^{10}	5.0×10^{7}
Human F	4.1×10^{10}	1.6×10^{7}
Human G	1.4×10^{11}	3.4×10^{7}
Human H	1.8×10^{10}	2.5×10^{7}
Mean	3.7×10^{10}	2.4×10^{7}

[a] enumerated on YN-17

[b] enumerated on M-Tec

TABLE D4

LEVELS OF SORBITOL-FERMENTING BIFIDOBACTERIA AND ESCHERICHIA COLI IN HUMAN FECES AS ISOLATED ON HBSA AND M-TEC

SAMPLE SOURCE	Microorganisms Per Gram of Feces	
	Bifidobacterium spp[a]	Escherichia coli[b]
Human A	2.3×10^7	1.8×10^7
Human B	1.2×10^7	2.0×10^7
Human C	3.2×10^7	1.6×10^7
Human D	4.0×10^6	9.4×10^6
Human E	2.2×10^6	5.0×10^7
Human F	4.2×10^7	1.6×10^7
Human G	1.9×10^7	3.4×10^7
Human H	3.0×10^7	2.5×10^7
Mean	2.0×10^7	2.3×10^7

[a] enumerated on HBSA

[b] enumerated on m-Tec

TABLE D5

MEAN SURVIVAL COUNTS OF BIFIDOBACTERIA AND ESCHERICHIA COLI IN LAKE ONTARIO WATER ENUMERATED OVER A MAXIMUM THREE DAY PERIOD

TEMPERATURE RANGE C	IDENTIFICATION CODE	TYPE OF ISOLATE OR SAMPLE	TIME (hrs)						
			0	2	5	12	24	48	72
21 - 23	EY-6	Sewage^{a}	2.8x10^{3}	9.6x10^{2}	1.5x10^{2}	8.2	ND	-	-
21 23	308	Fecal^{a}	7.5x10^{4}	1.1x10^{3}	9.5x10^{2}	11.7	0.3	ND	-
21 - 23	701	B. breve^{a}	3.3x10^{4}	4.0x10^{3}	6.2x10^{2}	12.7	0.23	ND	-
21 - 23	696	B. bifidum^{a}	1.0x10^{5}	2.2x10^{4}	1.3x10^{3}	1.8	ND	-	-
21 - 24	P-2	Feces^{a}	8.3x10^{5}	9.3x10^{5}	1.0x10^{6}	8.4x10^{5}	3.0x10^{4}	4.0x10^{2}	-
21 - 23	-	E. coli^{b}	1.4x10^{4}	4.2x10^{3}	2.5x10^{3}	7.6x10^{2}	4.7x10^{1}	0.45	ND

ND: not detectable

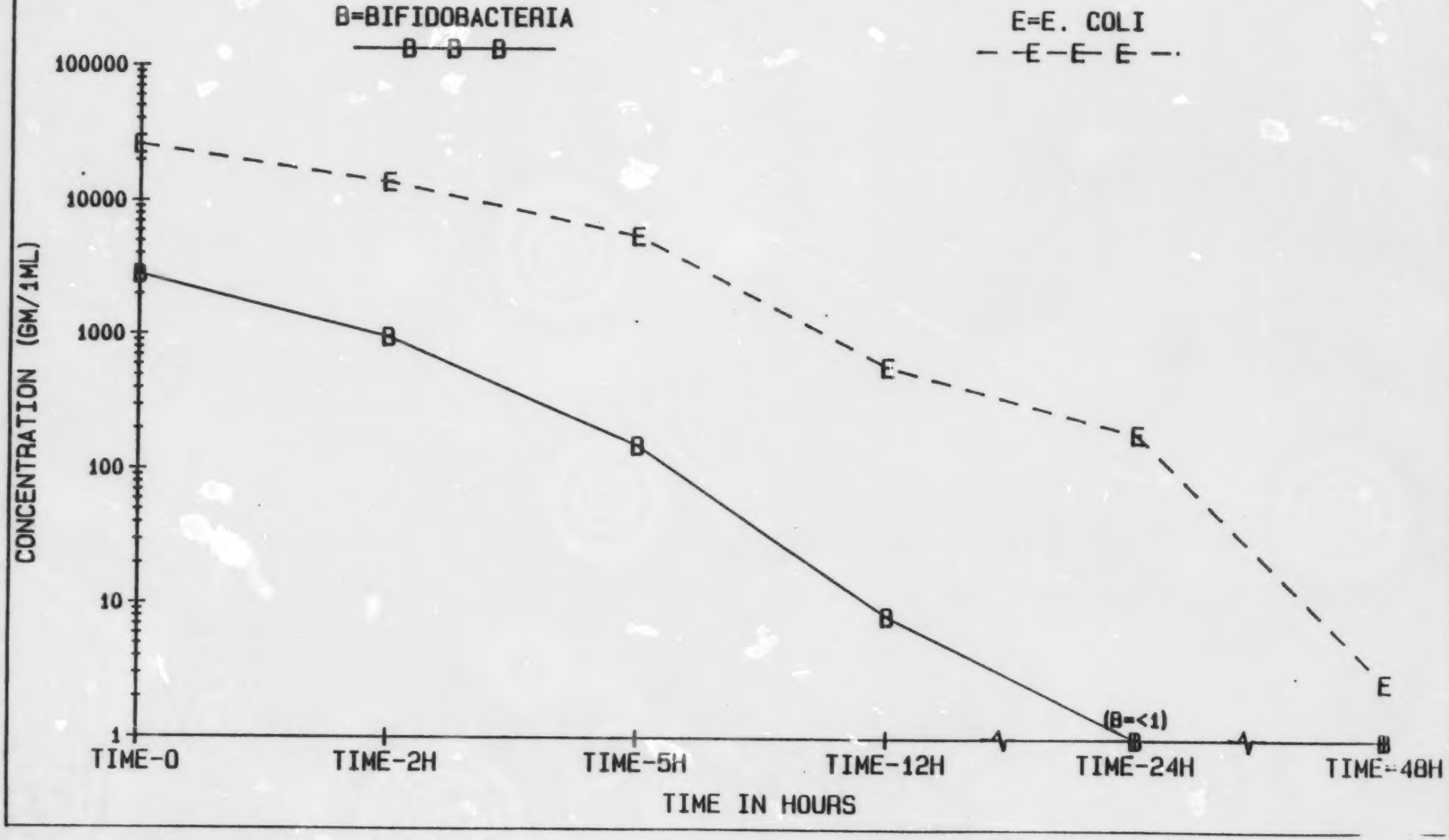
Note: all samples were performed in triplicate

^{a} : enumerated on YN-17

^{b} : enumerated on M-Tec

FIG.D2 IN-VITRO SURVIVAL OF BIFIDOBACTERIA ISOLATED FROM SEWAGE AND E COLI IN LAKE ONTARIO WATER

TEMPERATURE 21-23 DEGREES CELSIUS



second day of exposure. E. coli, in contrast, was able to survive as long as 48h after exposure to the environmental water, but was not detectable after the third day. After the 15h mark the bifidobacteria appeared to decline somewhat more rapidly than E. coli.

Pure Culture of Bifidobacteria Isolated from Human Feces

The bifidobacteria declined more quickly than E. coli within the first 2h of exposure with the bifidobacterial cultures decreasing greater than one order of magnitude and the E. coli cultures decreasing less than one order of magnitude (Fig. 3). Bifidobacterium cultures died off within 24h of exposure to the lake water in vitro.

Pure Culture of Bifidobacterium bifidum (ATCC #696)

The longevity of Bifidobacterium bifidum was relatively consistent with that observed with the E. coli culture. However, this pattern appeared to change after 5h where the B. bifidum decreased in numbers much faster than E. coli. After 24h, B. bifidum could only be isolated from one chamber in low numbers (i.e. < 1/1mL). (Fig. 4).

Pure Culture of Bifidobacterium breve (ATCC #701)

Bifidobacterium breve presented a more rapid decrease in density for the most part of the survival analysis when compared to E. coli. Decline of B. breve was more marked after 5h of exposure and the species could not be recovered after 24h. (Fig. 5).

Fecal Sample

Both Bifidobacteria and E. coli appeared to survive maintaining fairly steady densities between 0 and 12h (Fig. 6). However, rapid decline of the bifidobacteria occurred after this time interval whereas E. coli seemed to decrease in numbers much more slowly.

FIG. D3 IN-VITRO SURVIVAL OF BIFIDOBACTERIA ISOLATED HUMAN FECES AND E. COLI IN LAKE ONTARIO WATER

TEMPERATURE 21-23 DEGREES CELSIUS

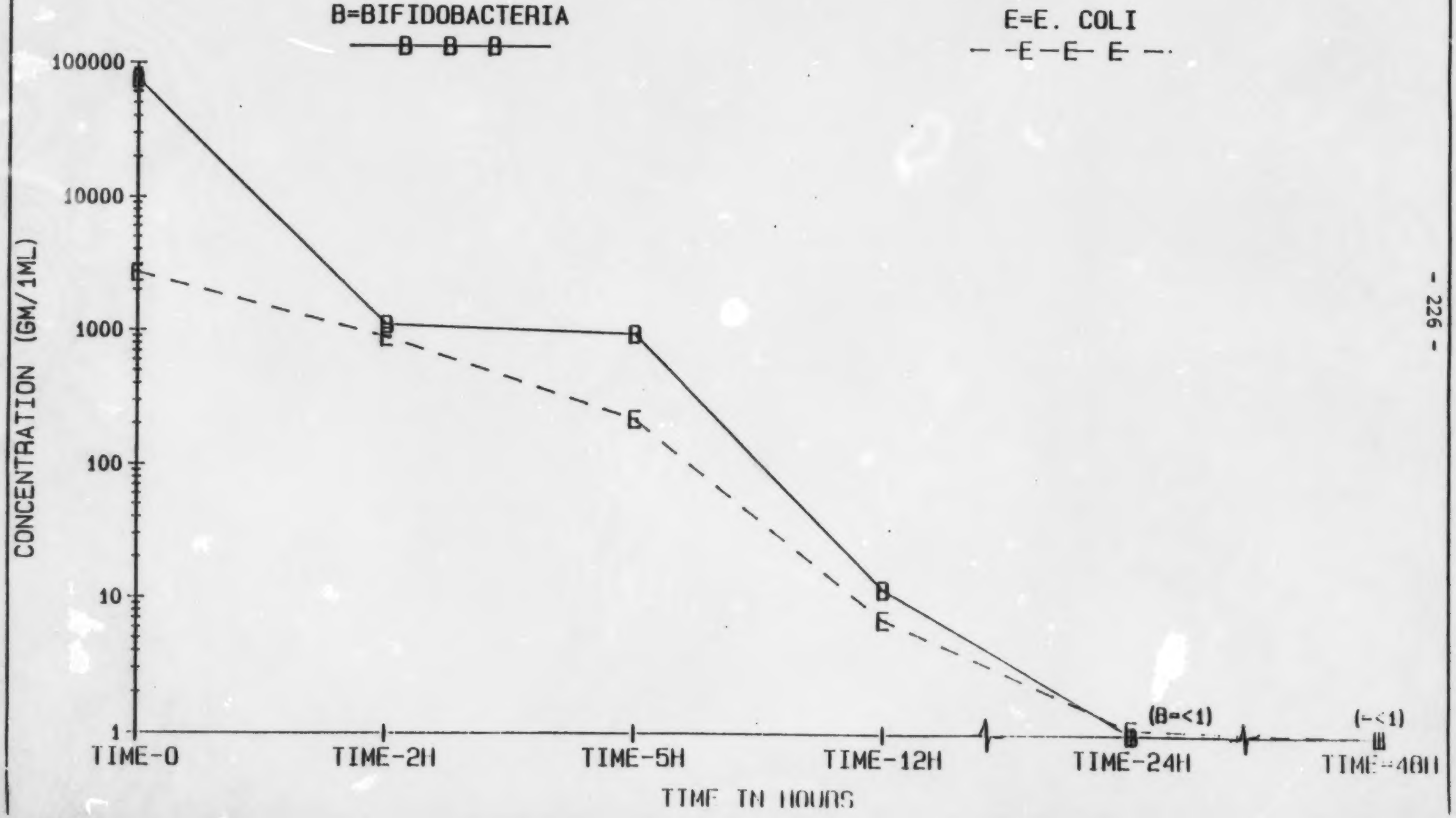


FIG. D4 IN-VITRO SURVIVAL OF BIFIDOBACTERIUM BIFIDUM (ATCC # 696) AND E COLI IN LAKE ONTARIO WATER

TEMPERATURE 21-23 DEGREES CELSIUS

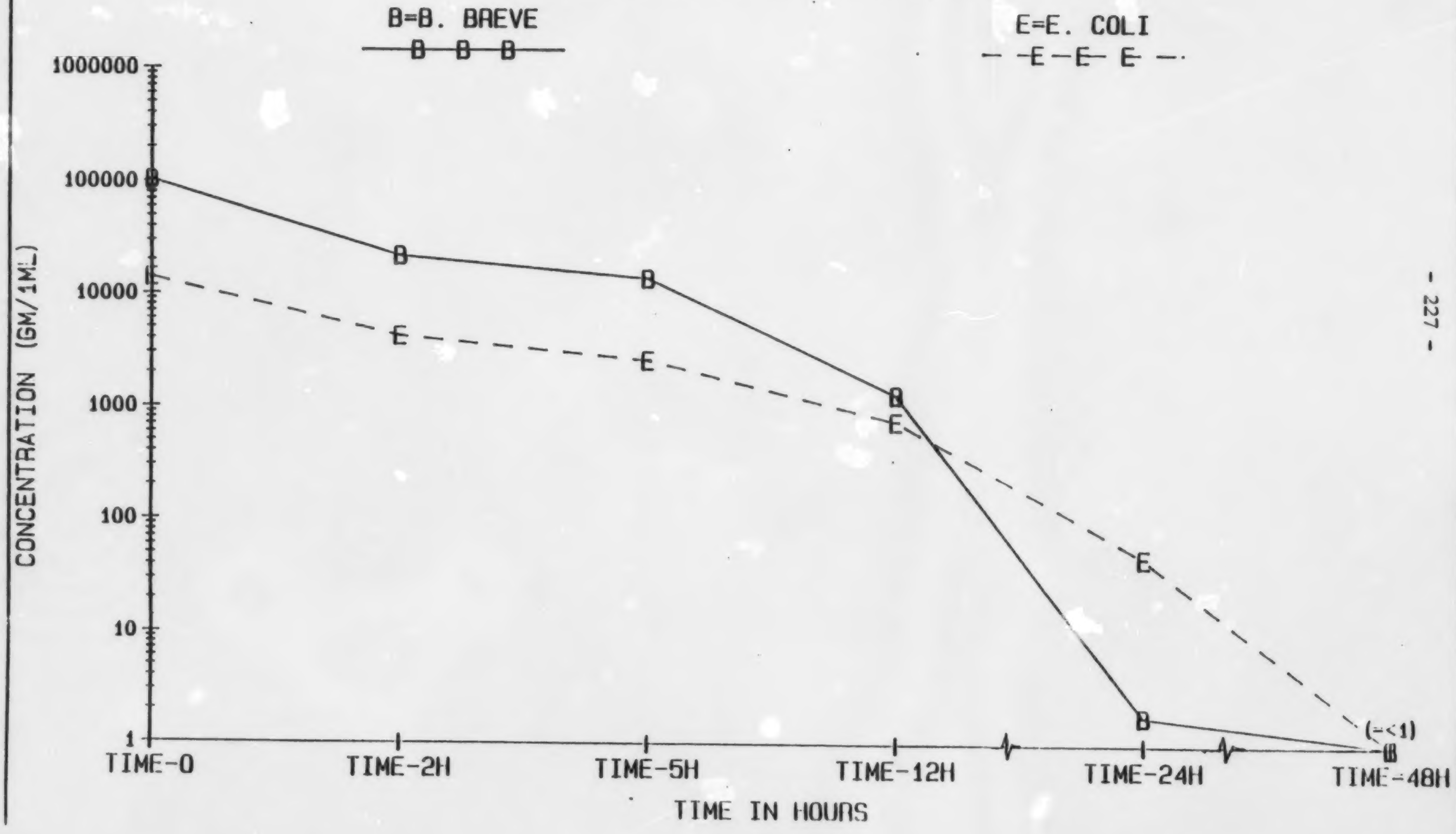


FIG. D5 IN-VITRO SURVIVAL OF BIFIDOBACTERIUM BREVE (ATCC # 701) AND E COLI IN LAKE ONTARIO WATER

TEMPERATURE 21-23 DEGREES CELSIUS

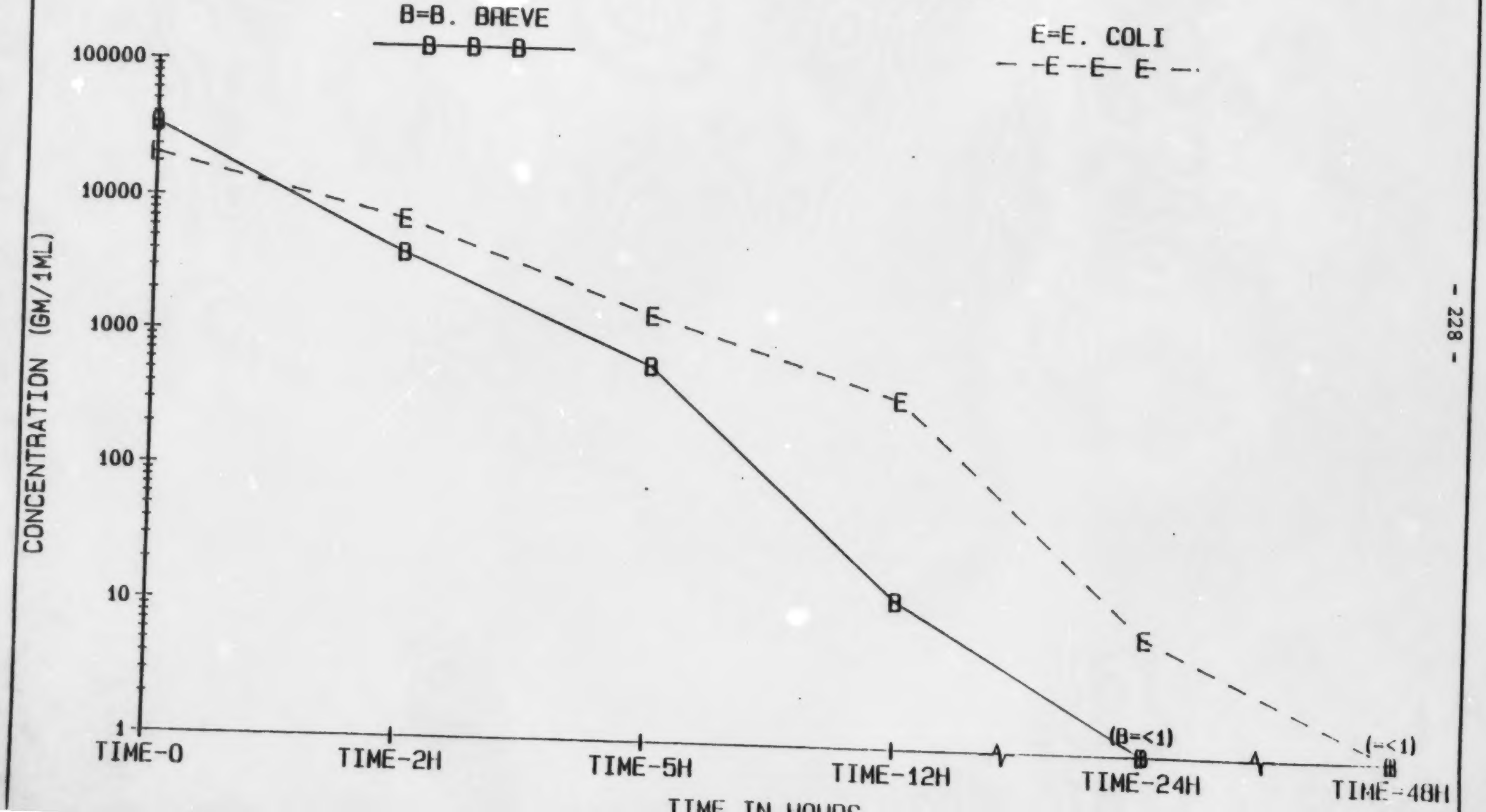
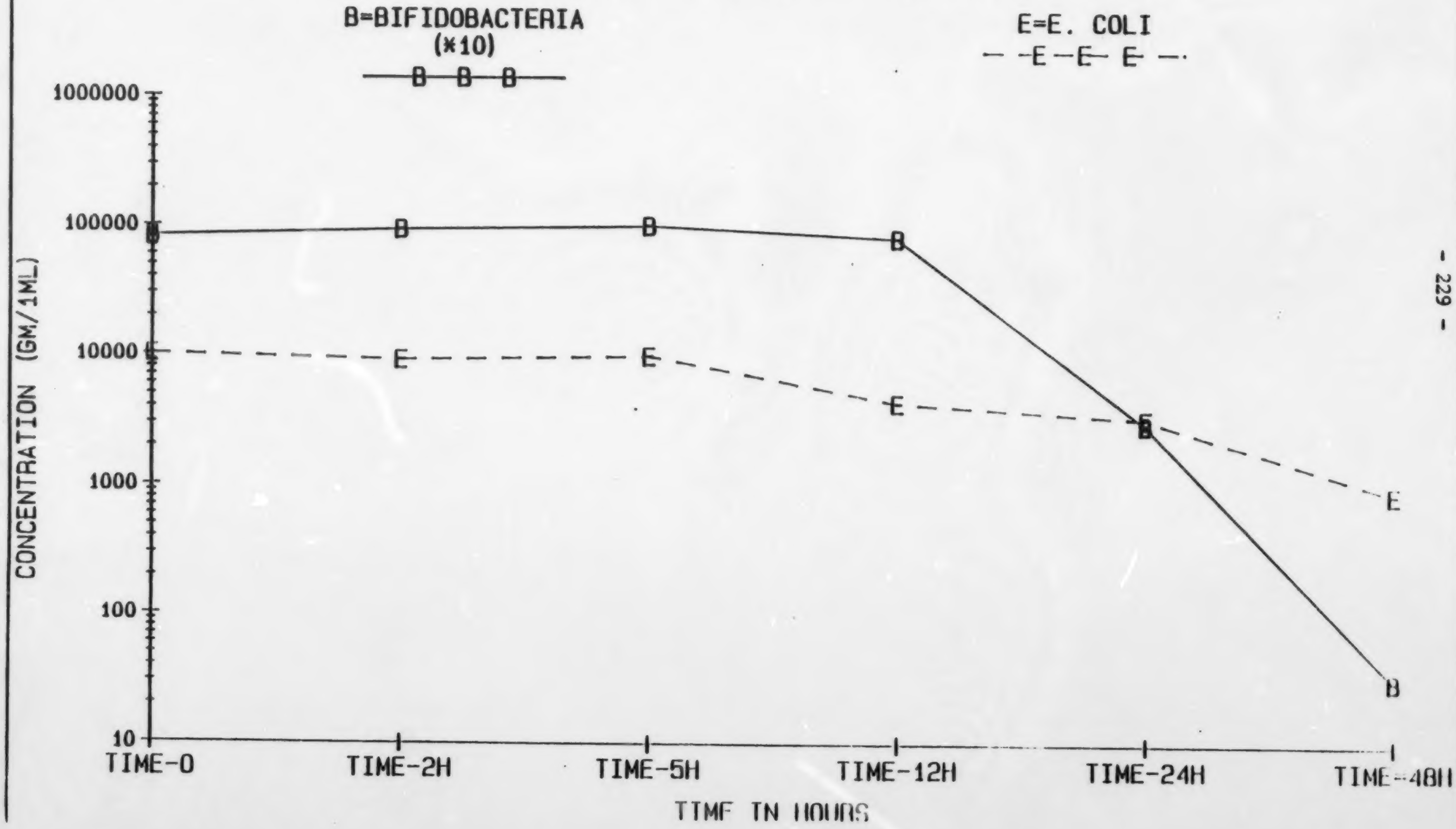


FIG. D6

IN-VITRO SURVIVAL OF FECAL BIFIDOBACTERIA AND E. COLI
IN LAKE ONTARIO WATER
TEMPERATURE 21-24 DEGREES CELSIUS



DISCUSSION

Part 1: Isolation and Enumeration

One of the major problems in assessing an organism's potential role as a water quality indicator stems from difficulties in its isolation. Therefore a comparison of the three media: YN-17, MRS, and MPN for their selectability and specificity was performed.

Although visible target colonies were observable on MRS after a relatively short incubation time (i.e. 24h), the medium was not found to be sufficiently specific for bifidobacteria. The MRS medium was prepared utilizing the antibiotic cocktail devised for YN-17 (Appendix 2). However, this formulation was not able to suppress the occurrence of excessive contamination by lactobacilli and fecal streptococci. This occurrence is best explained by the fact that the MRS medium was richer in nutrient content than the other two media, having been originally developed to support the growth of Lactobacillus spp. (Dept. of Nutrition, University of Toronto, personal communication). Although MRS was not appropriate for the specific isolation of bifidobacteria, it was utilized (void of antibiotics), in broth tubes to support the growth of pure cultures of these organisms. MRS, as a nutrient medium, was found to be suitable for bifidobacteria with visible growth appearing as early as 16h after incubation.

YN-17 and MPN appeared to be equally selective for bifidobacteria as seen in Table 2. The results stated previously show that the two media were also comparatively specific for bifidobacteria where background fecal streptococci were found in levels no higher than 5% on either media. However, as it was earlier stated, a potential water quality indicator should possess a relatively rapid means of detection and enumeration (Bonde, 1978). In this regard, YN-17 was superior to MPN in that YN-17 required a 24h shorter incubation period in comparison to MPN.

Fecal streptococci were found to be the most successful competitor to bifidobacteria where on occasion, the former would overgrow the latter on YN-17. Hence a means to better suppress the growth of fecal streptococci on this medium was sought. Attempts to suppress fecal streptococci contamination using 0.3% and 0.4% LiCl in YN-17 failed. Fecal streptococci contamination prevailed and were found in numbers consistent with those obtained prior to the incorporation of LiCl.

The use of E. coli as a water quality indicator of recent fecal contamination (Seyfried et al., unpublished report) provides a standard with which to compare bifidobacteria. For the reasons mentioned above, YN-17 was the medium of choice for the enumeration of bifidobacteria from feces and subsequent density level comparisons with E. coli. Also employed was Mara and Oragui's (1983) HBSA medium for the enumeration of sorbitol-fermenting bifidobacteria.

The mean density levels of bifidobacteria and E. coli found in the feces of eight human adults have been tabulated (Table 3). Similarly, the density levels of sorbitol-fermenting bifidobacteria in comparison with E. coli have been presented in Table 4. The results of this study showed bifidobacteria to exist in feces at a 1000-fold higher concentration than E. coli. The sorbitol-fermenting species of bifidobacteria presented themselves in numbers comparable to those of E. coli (i.e. 10^7) in the sample tested. These values were consistent with the bacterial content, found in human feces, reported by Mara and Oragui (1983).

Part 2: Survival Study

Figures 2-6 represent the results obtained form the survival study. In every case both bifidobacteria and E. coli were found to decline after exposure to Lake Ontario water, in vitro. Possible reasons for the observed die-off include: nutrient depletion, diffusion of nutrients out of the dialysis chambers, toxic chemical diffusion into the chamber, and where bifidobacteria is concerned, the effects of atmospheric oxygen levels toxic to the anaerobes.

Pure cultures of bifidobacteria appeared to parallel the die-off trends in E. coli up to the 5h mark; thereafter bifidobacteria began to show a slightly more rapid decline (Figures 2, 4, 5). In all cases bifidobacteria died off within 24h of exposure whereas E. coli persisted as long as 48h.

As was noted, the bifidobacteria survival was comparable with E. coli within the first 5h of exposure. It is suggested that, upon exposure to the environment, some of the bifidobacteria were able to utilize nutrients from dead cells to maintain their existence. Eventually as time passed the effects of diffusion, resulting in dilution of nutrients and exposure of the pure culture to any toxic chemicals present in the water, may have had an impact on the survival of the organisms. Also, it is expected that competition for nutrients increased over time due to the depletion of nutrients by the organisms themselves. The more rapid decline of bifidobacteria in comparison to E. coli may possibly be accounted for by considering oxygen diffusion into the chamber. Initially the pure cultures were suspended in autoclaved environmental water (see Methods and Materials). Heating of the water would have led to a subsequent driving-off of oxygen. Once the chambers containing the suspension of bifidobacteria were placed into the lake water, oxygen content may have increased due to diffusion. Thus it was perhaps the toxic levels of oxygen after the 5h mark that lead to the more rapid decline of the bifidobacteria at this point.

Figure 3 presents bifidobacteria and E. coli in fairly steady decline with both cultures becoming undetectable within 24h. The more rapid decline of both organisms in comparison to those cultures presented in Figures 2, 4 and 5, may have been the result of differences in environmental water utilized in the study. Table 1 shows that the water used for the cultures (Fig. 3) was retrieved from a boat dock. It is quite possible that this water contained residual chemicals, emitted by boat engines, that would have a toxic effect on the bacterial cultures.

Results of exposure of a mixed culture of bifidobacteria are presented in Table 6. In this instance, a diluted fecal sample was introduced into the dialysis chamber in an effort to present bifidobacteria and E. coli in vitro, as they might be found in the natural environment.

As can be seen in Figure 6, both organisms tended to exist in a relatively stationary state over a 12h period. These results are inconsistent with the survival data found using the pure cultures. However, it would be plausible to state that the greater nutrient level provided by the diluted fecal material within the chamber, allowed the organisms to maintain their numbers.

It is possible that competition with other organisms found in human feces, nutrient depletion, and effects of toxic chemicals resulted in the decline of the bifidobacteria and E. coli after 12h exposure to the environment.

Once again the more rapid decline phase of bifidobacteria is suggested to have been due to the fact that the organisms are strict anaerobes.

CONCLUSIONS

Comparisons with *E. coli*, presently being used as a water quality indicator of recent fecal pollution (Seyfried *et al.*, unpublished report), support the assumption that sorbitol-fermenting species of bifidobacteria may serve as indicators of recent fecal contamination in surface waters. As was shown in this study, their densities in feces, their ability to survive *in vitro* for as long as 24 hours, and the existence of methods allowing for their selection and recovery support this proclamation.

Unfortunately an *in vitro* survival study poses some limitations. The effects of ultraviolet radiation, temperature changes, drying, predation, competition, water flow, and pH changes occurring in the natural environment could never be mimicked completely *in vitro*.

Nonetheless, the fact that certain species of bifidobacteria have been found to be restricted to human feces, and their tendency to die off quite rapidly make these organisms promising water quality indicators for determining pollution sources and locations.

RECOMMENDATIONS

1. A study should be performed in Ontario surface waters to characterize the survival trends of bifidobacteria *in situ*.
2. An epidemiological study should be performed in Ontario to confirm the statements that sorbitol-fermenting species of bifidobacteria are restricted to humans.

APPENDIX 1

1. **Phosphate Solution/Dilution Blanks:**

   a) Dissolve 34.0g KH_2PO_4 in 500mL dH_2O. Adjust pH to 7.2. Dilute to 1L with dH_2).
   b) Dissolve 50g $MgSO_4$ in 1L dH_2O.

2. **Magnesium Chloride Solution:**

$MgCl_2$	38.0g
Distilled water (dH_20)	1.0L

Stir ingredients to dissolve. Autoclave for 15min. at 121° C (15lbs pressure).

3. **Dilution Blanks:**

Phosphate Solution (1)	1.25mL
MgCl Solution (2)	5.0mL

Mix solutions and dispense 99mL (plus 4mL for evaporation) into dilution blanks. Autoclave for 15min. at 121° C (15lbs pressure).

4. **Gelatin Phosphate Buffer:**

gelatin	2g
Na_2HPO_4	3.7g
NaH_2PO4	7.25g
dH_2O	1.0L

Dissolve in 500mL of distilled water. Adjust pH to 7.2 ±0.2 with 1.0N NaOH. Dissolve gelatin in 500mL of distilled water (heat to < 90° C). Autoclave for 15min. at 121° C (115lbs pressure).

5. **Urease Reagent:**

Urea	10.0g
Phenol Red	0.05g
Ethanol	0.5mL

Mix ingredients and adjust pH to 5.0±0.2.

APPENDIX 2

Growth Media

1. MRS (oxoid) broth was prepared according to the manufacturer's recommendations.

2. MRS selective medium was prepared by addition of th YN-17 antibiotic cocktail (4a) and 20g of BactoAgar.

3. m-TEC Agar:

Proteose peptone #3	5.00g
Yeast extract	3.00g
Lactose	10.00g
NaCl	7.50g
K_2HPO_4	3.3g
KH_2PO_4	1.00g
Sodium lauryl sulphate	0.20g
Sodium deoxycholate	0.10g
Bromocresol purple	0.08g
Bromocresol red	0.08g
Agar	15.00g
dH_2O	1.00L

Mix above ingredients and heat to 90° C to dissolve. Autoclave for 15min. at 121° C (15lbs pressure). Cool to 50° C and dispense in sterile petri dishes. Final pH 7.1±0.1.

4a. YN-17

Yeast extract	20.0g
Polypeptone (BBL)	10.0g
Lactose	10.0g
Casamino acids	8.0g
NaCl	3.2g
Bromocresol green	0.3g
Cysteine hydrochloride	0.4g
BactoAgar	15.0g
dH_2O	1L
Antibiotics:	
Nalidixic acid	0.03g
Kanamycin sulphate	0.05g
Polymixin B	0.006g

Mix above ingredients with the exception of the antibiotics and heat to 90° C to dissolve. Autoclave for 15min. at 121° C (15lbs pressure). Cool to 50° C and add antibiotics listed above. Dispense in sterile petri dishes. Final pH 6.9±0.2.

b. YN-17(-):
Same formulation as above with the exclusion of antibiotics.

5. Human Bifid Sorbitol Agar (HBSA):

Sorbitol	10.0g
Polypeptone (BBL)	10.0g
Yeast extract	20.0g
Casamino acids	8.0g
NaCl	3.2g
Bromocresol purple	0.1g
Cysteine hydrochloride	0.4g
Bacto Agar	15.0g
dH_2O 1.0L	
Antibiotics:	
Nalidixic acid	0.03g
Kanamycin sulphate	0.05g
Polymixin B	0.0012g

Mix above ingredients with the exception of the antibiotics and hat to 90° C to dissolve. Autoclave for 15min. at 121° C (15lbs pressure). Cool to 50° C and add antibiotics listed above. Final pH 6.8±0.2.

6. MPN:

a)	Lactose	20.0g
	$(NH_4)_2SO_4$	5.0g
	K_2HPO_4	1.0g
	Tween 80	1.0g
	Bromocresol purple	0.0016g
	Bacto Agar	20.0g

Salt Solution: (5.0mL)

$FeSO_4*7H_2O$	0.5g
$MnSO_4*2H_2O$	0.4g
$MgSO_4*7H_2O$	10.0g
NaCl	0.3g
dH_2O	250mL

b)	Biotin	0.0001g
	Pantothenic acid	0.002g
	Riboflavin	0.001g
	Adenine	0.001g
	Guanine	0.001g
	Xanthine	0.001g
	Uracil	0.001g

Mix ingredients (a) in 740mL dH_2O and heat to 90° C to dissolve. Autoclave for 15min. at 121° C (15lbs pressure). Cool to 50° C. Mix ingredients (b) in 10mL dH_2O and sterilize by membrane filtration. Add (b) to (a) and adjust pH to 6.8±0.2.

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SPECIAL NOTES

There seemed to exist some discrepancies between the findings of this study and those of Mara and Oragui (1983) in regards to YN-17 medium. Mara and Oragui found flat, non-mucoid colonies measuring 2-4mm and dark green in colour to be background streptococci. They distinguished domed, mucoid colonies measuring 1-2mm and pale green with a pale green periphery as being bifidobacteria.

However, this study showed the opposite to be true. This is to say the smaller dark green/blue flatter colonies were identified as bifidobacteria and those exhibiting a pale green periphery were contaminating streptococci or more likely a mixture of bifidobacteria and streptococci. Any colonies pale green in colour that did not contain a darker centre were found to be streptococci.

APPENDIX E

Clostridium perfringens and Bifidobacterium sp. as Tracers in Storm Sewers

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METHODS

Sample Collection

1. High priority storm sewage-triplicate samples from three in-line sample points.
2. Sanitary sewage-triplicate samples from three in-line sample points.
3. Non-priority storm sewage-triplicate samples from two in-line sample points.
4. Fecal samples from humans, cats, dogs.

Analysis

Membrane filtration for parameters:

Fecal coliforms/E. coli

m-TEC + urease treatment
44.5 ± 0.5° C; 23 ± 1 hr
(Dufour, Strickland and Cabelli, 1975)

Clostridium perfringens

Heat treatment at 70° C for 30 minutes
mCP-2 at 37° C for 48 hr
(Ontario Ministry of the Environment, 1986)

Bifidobacterium sp.

YN17 at 37° C for 48 hr, anaerobic gas pack, anaerobic jar
(Mara and Oragni, 1983)
isolates tested in sorbitol broth)

Table E-1

Ratio of *E. Coli* to *Clostridium perfringens* and of Bifidobacteria to *E. Coli* for High Priority and Non-Priority Storm and Sanitary Sewage

Sewer Sample	E/C
HP Storm A	48
HP Storm B	141
HP Storm Y	5.3
HP Storm C	7.9
NP Storm X	7.5
NP Storm Z	-
Sanitary D	83
Sanitary E	50
Sanitary F	150

E/C = *E. Coli*: *Clostridium perfringens*

Sewer Sample	B/E
HP Storm A	1.7
HP Storm B	2.3
HP Storm Y	1.9
HP Storm C	0.25
NP Storm X	0.01
NP Storm Z	0.04
Sanitary D	0.02
Sanitary E	0.02
Sanitary F	0.05

B/E = Bifidobacterium/*E. Coli*

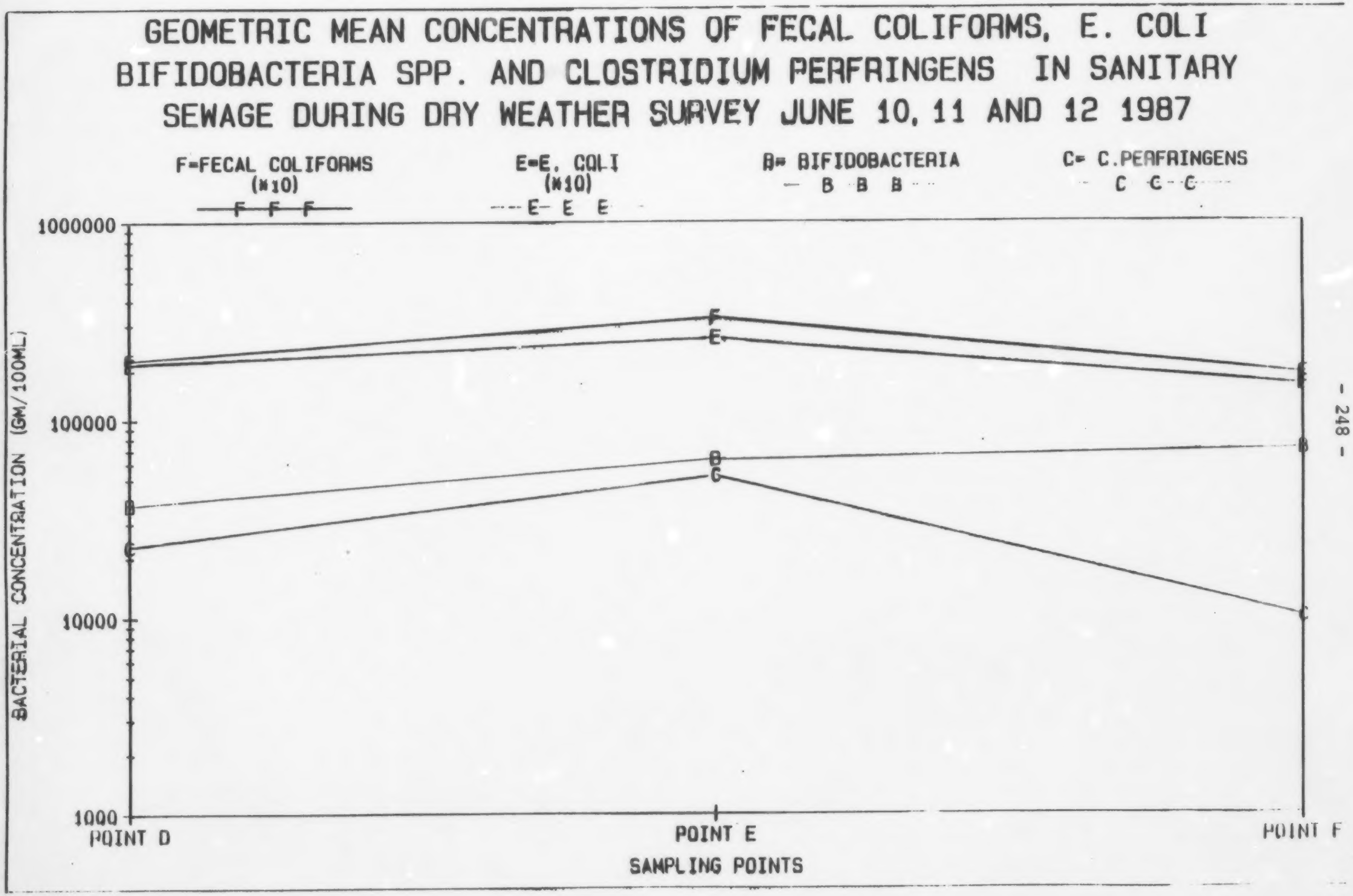
Table E-2

Geometric Mean Concentrations of Fecal Coliforms, _E. Coli_, _Clostridium_ _perfringens_ and Bifidobacteria in High Priority, Non-Priority Storm and Sanitary Sewage

Sewer Sample	Fecal Coliforms	_E. Coli_	Bifidobacteria	_C. perfringens_
HP Storm A	4.9×10^4	3.1×10^4	5.2×10^4*	6.4×10^2
HP Storm B	8.5×10^4	6.2×10^4	1.4×10^5*	4.4×10^2
HP Storm Y	3.8×10^3	2.9×10^3	5.6×10^3*	5.4×10^2
HP Storm C	9.3×10^3	6.0×10^3	1.5×10^3	7.6×10^2
NP Storm X	2.0×10^3	9.0×10^2	1.2×10^1*	1.2×10^2
NP Storm Z	4.1×10^4	1.1×10^4	4.4×10^2	-
Sanitary D	2.0×10^6	1.9×10^6	3.7×10^4	2.3×10^4
Sanitary E	3.3×10^6	2.6×10^6	6.3×10^4	5.2×10^4
Sanitary F	1.7×10^6	1.5×10^6	7.1×10^4	1.0×10^4

* Approximate value.

Figure E-1



GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, E. COLI BIFIDOBACTERIA SPP. AND CLOSTRIDIUM PERFRINGENS IN SANITARY SEWAGE DURING DRY WEATHER SURVEY JUNE 10, 11 AND 12 1987

Figure E-2

GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, E. COLI BIFIDOBACTERIA SPP. AND CLOSTRIDIUM PERFRINGENS IN H.P. STORM SEWAGE DURING DRY WEATHER SURVEY JUNE 10, 11 AND 12 1987

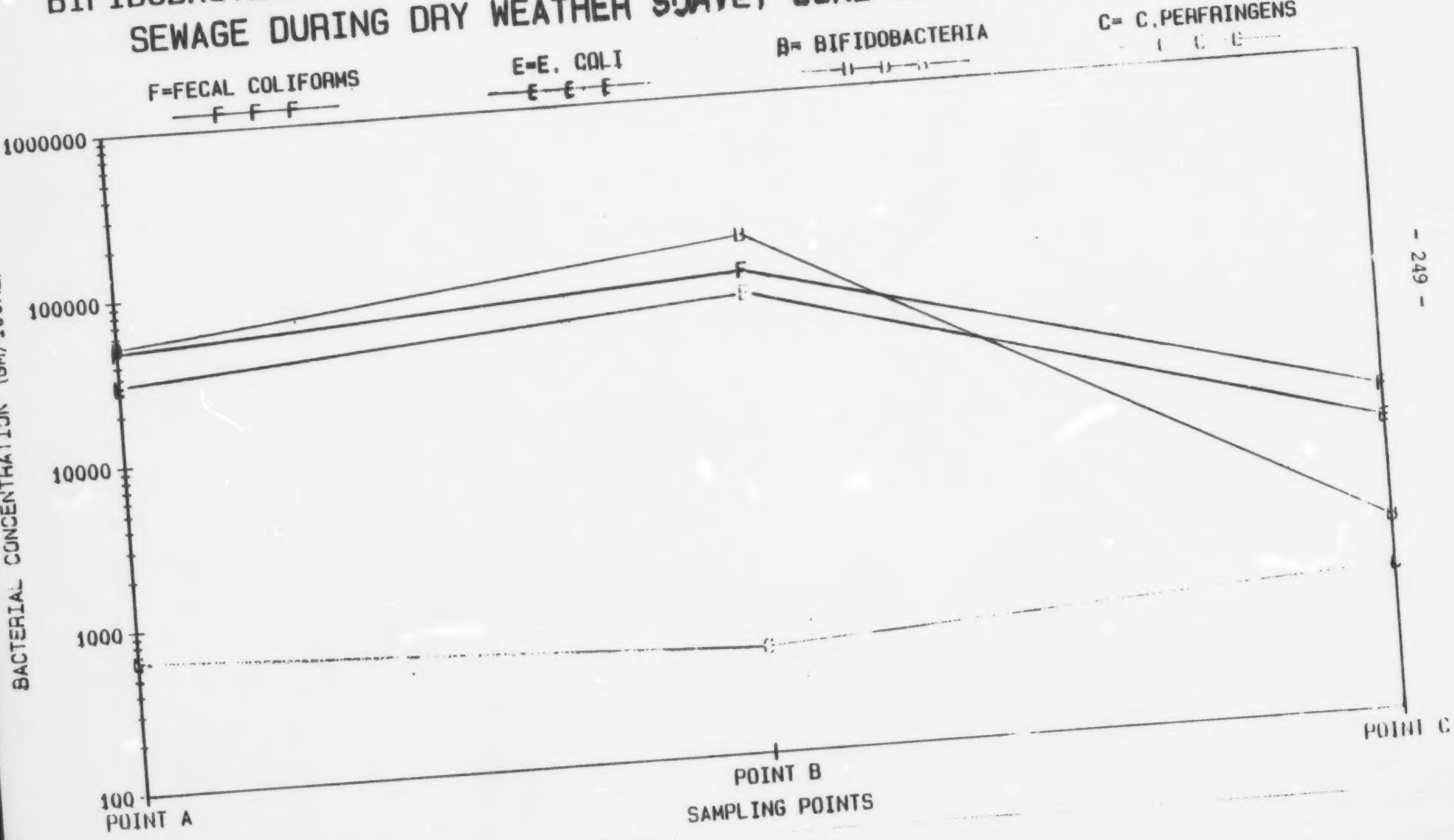


Figure E-3

A COMPARISON OF THE GEOMETRIC MEAN CONCENTRATIONS OF E. COLI, CLOSTRIDIUM PERFRINGENS AND BIFIDOBACTERIA IN HIGH PRIORITY AND NON PRIORITY STORM AND SANITARY SEWAGE (LOG. VALUES)

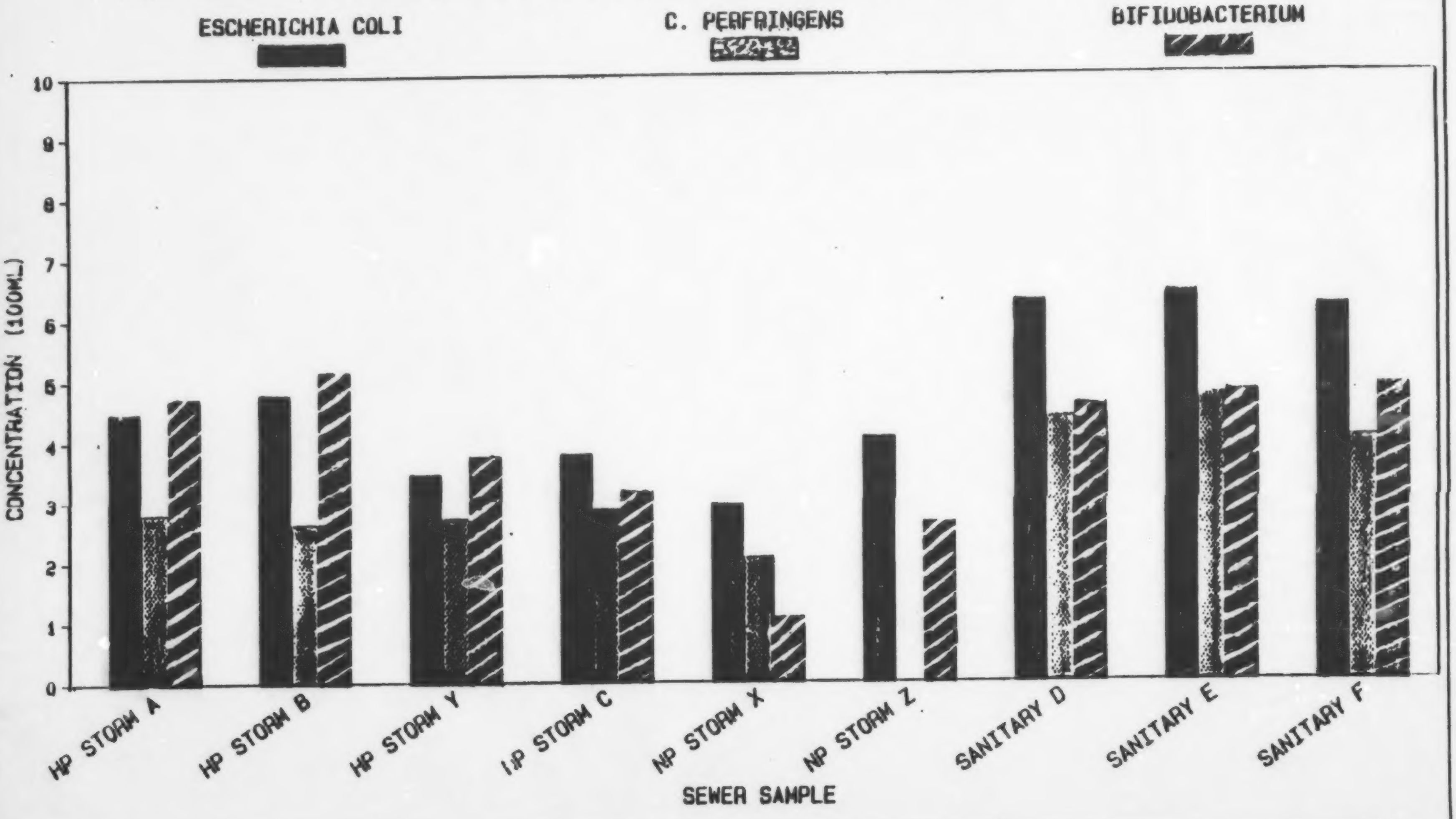


Figure E-4

A COMPARISON OF THE GEOMETRIC MEAN CONCENTRATIONS OF E. COLI, CLOSTRIDIUM PERFRINGENS AND BIFIDOBACTERIA IN HUMAN, CAT AND DOG FECES (LOG. VALUES)

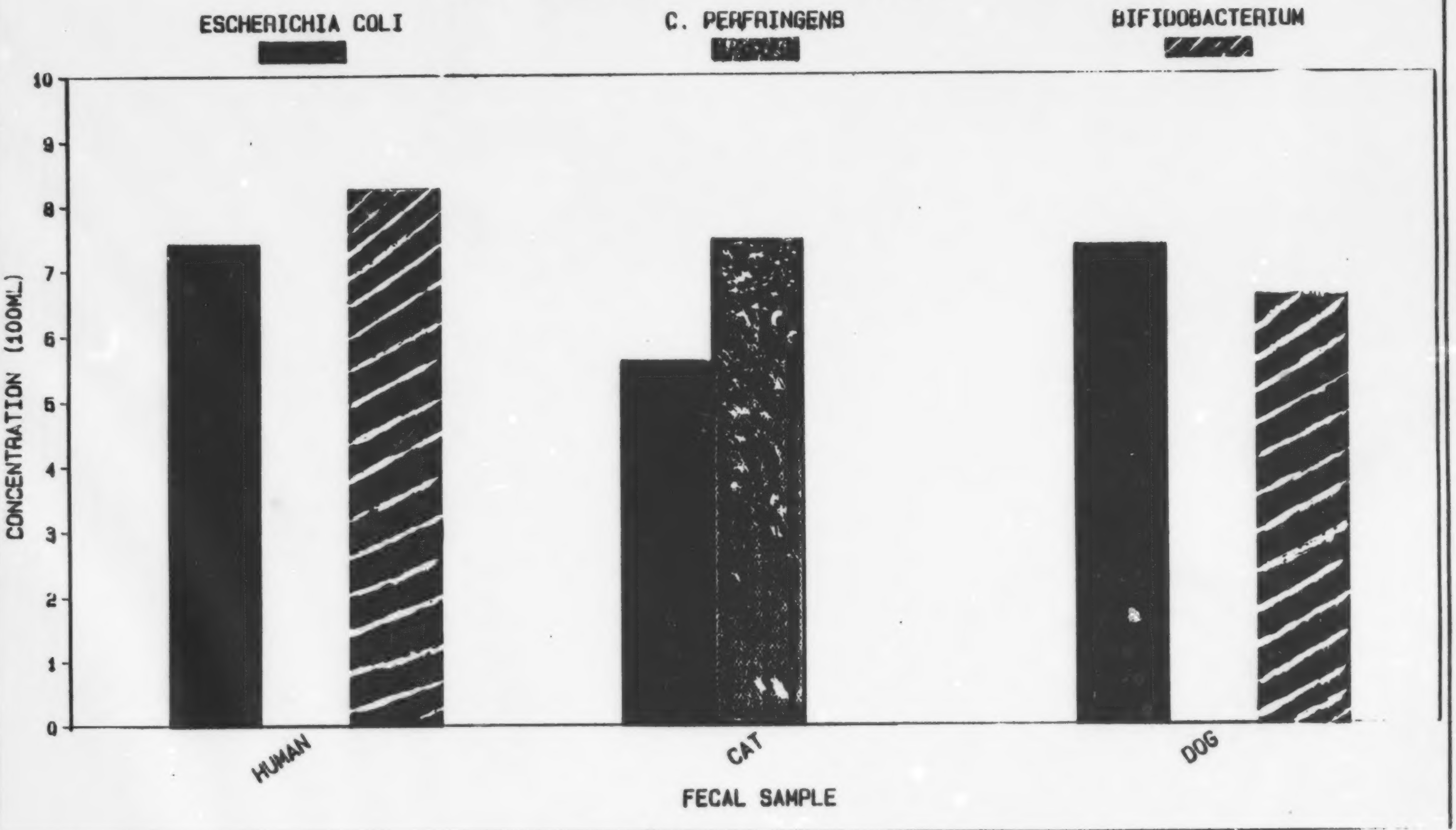
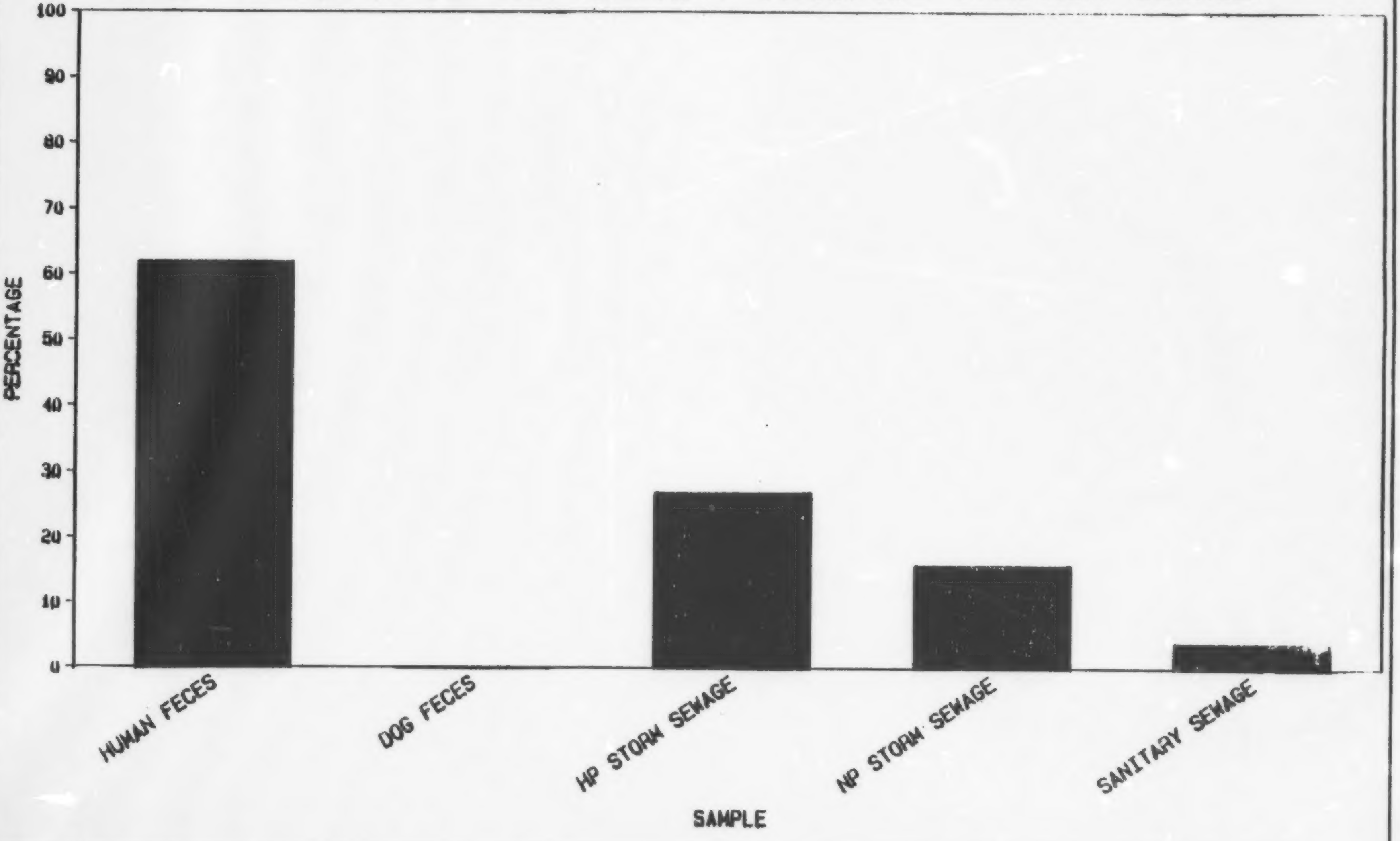


Figure E-5



PERCENTAGE OF SORBITOL FERMENTING BIFIDOBACTERIA IN FECES, HIGH PRIORITY AND NON PRIORITY STORM AND SANITARY SEWAGE.

SUMMARY

Clostridium perfringens

- present in feces in high density
- good survival characteristics
- no other source in the environment
- expensive to recover
- no human/non-human determination possible
- indication of age of pollution
- indication of water treatment performance

Bifidobacterium spp.

- indication of recent pollution
- human/non-human determination possible
- no other source in the environment
- present in feces in high concentration
- no replication extraintestinally
- easy to identify

APPENDIX F

CHARACTERIZATION OF PSEUDOMONAS AERUGINOSA
FROM STORM AND SANITARY SEWERS

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INTRODUCTION

Increased concern for public health hazard has lead to methods aimed at reducing the input of fecal pollution in surface water bodies. Human fecal material in sanitary sewage, contains bacteria which may cause infections in the appropriate host. Under normal circumstances, this sanitary sewage is treated in wastewater treatment plants, reducing bacterial numbers, and the effluent is discharged into natural waterways. This process serves to minimize the contact between humans and human fecal material thereby decreasing the probability of infection.

Recently, the Toronto Area Watershed Management (TAWM) has shown that storm sewers contribute to the fecal pollution of the Humber and Don Rivers. Originally these sewers were designed for channelling storm water from urban areas into surface waters to avoid the flooding that would have resulted, as a consequence of the blocking of natural flow patterns that existed before urbanization. The bacterial content of storm sewers should be similar to direct runoff and have virtually no human fecal input.

High fecal bacterial levels detected in storm sewers suggests that these sewers may be contaminated with human fecal wastes. The problems resulting from this are obvious: if sewage enters the surface water supplies untreated, the probability of infecting a suitable host is high. Therefore, in order to determine which home connections are contributing to fecal pollution, some method of tracing the pollution must be developed.

One opportunistic pathogen that has been recovered from human fecal material and sewage is Pseudomonas aeruginosa. Ringen and Drake (1952) have shown that even though Pseudomonas aeruginosa can be isolated from a wide variety of natural sources, its distribution is limited. They found that

Pseudomonas aeruginosa was not isolated in natural waters of mountain streams or deep wells remote from human habitation and free of human waste material. In addition Wheater and coworkers (1978) have shown that Pseudomonas aeruginosa was not found in a variety of animal feces. The presence of this organism in barnyards, animal feces and soil was due to chance and close association with man. Therefore, the presence of Pseudomonas aeruginosa in surface waters and storm sewage may indicate the presence of human sanitary wastes.

Subspeciation of Pseudomonas aeruginosa has classified this microorganism into 17 different serotypes according to their somatic-O-antigen. Studies by Seyfried and Fraser (1977) and Young and Moody (1974) have shown that tracing the Pseudomonas aeruginosa infection in clinical and environmental settings is possible. Therefore, by adopting their methods of pollution source tracings in storm sewers, one may be able to determine the presence of human sanitary waste in storm water and trace the location of the inputs.

With a limited amount of information on the distribution of serotypes of Pseudomonas aeruginosa and their distribution in the environment, work must be performed to determine if there is a difference between serotypes of Pseudomonas aeruginosa found in humans and those from environmental sources. If a serotype, specific to sanitary wastes, if found, then isolating the same serotype from storm sewers might indicate the presence of sanitary waste.

Another method which may prove valuable in determining if a specific type of Pseudomonas aeruginosa is associated with human feces, is restriction enzyme analysis. This method may be able to determine if a specific Pseudomonas aeruginosa genotype is present in human fecal material as compared to environmental sources. This in turn may serve as the probe used to determine the presence of human Pseudomonas aeruginosa contamination in storm sewers.

OBJECTIVES OF RESEARCH

The objectives of this research were to:

1. Characterize isolates of Pseudomonas aeruginosa from sanitary and storm sewage according to their serotypes and genotypes; in order to see what differences and/or similarities exist among isolates obtained from these types of waste and to apply this knowledge to develop a method of detecting the presence of sanitary waste in storm sewage.
2. Determine which method (serotyping or genotyping) will give more discriminating information about a specific strain of Pseudomonas aeruginosa.

MATERIALS AND METHODS

3.1 Sample Collection

Samples were collected from three different points along the storm sewer line, in triplicate, over a three day period.

Samples were also collected at three points along the sanitary sewer lines.

Water samples were collected in sterile Sodium Thiosulphate EDTA treated bottles (Appendix) and transported to the laboratory on ice. Upon receipt of the samples, analysis was performed within three hours.

3.2 Enumeration of Pollution Indicator Organisms

Appropriate dilutions, ranging from 50ml to 10^{-4}, of each water sample were made in phosphate solution (Appendix). Water samples were analyzed using standard membrane filtration techniques (APHA, 1989). Water samples were filtered through 0.45 μm porosity membranes (Gelman Sciences) and subsequently placed on selective media. Table 1 lists the media, incubation times and temperatures used for quantitation of different bacterial groups.

3.3 Isolation of Pseudomonas aeruginosa

Approximately 10 bacterial colonies were picked form m-PA plates, after the incubation period, and streaked onto nutrient agar (Difco) plates for isolated colonies. Plates were incubated at 35° C for 24 hours. One isolated colony was picked from the nutrient agar plates and incubated at 35° C for 24 hours. Cultures were maintained on BHI (Difco) slants overlaid with sterile paraffin oil. These slants were maintained at 20° C.

3.4 Biochemical Tests Performed on Pseudomonas aeruginosa

An inoculum of the culture was removed from the BHI slant and streaked on BHI agar plates for two consecutive transfers. Plates were incubated at 35° C for 24 hours.

1. - 3% KOH Test

The 3% KOH test (Fluharty and Packard, 1967) was used to determine the gram reaction of each isolate. A small inoculum of the culture was mixed with a drop of 3% KOH solution on a slide. The suspension was observed for consistency.

Gram-positive organisms did not gel when mixed with KOH while gram-negative organisms (Pseudomonas aeruginosa) form a thick stringy gel. Results were recorded immediately.

TABLE 1: Media and Incubation Parameters for Enumerating Selected Bacterial Groups

Bacterial Group	Medium[a]	Incubation Temperat. °C	Time (hrs)	Target colony Morphology
Pseudomonas aeruginosa	m-PA	41.5	48	-flat colonies with an irregular edge -may be brown, green, tan or gold in color
Escherichia cdi	m-TEC-MUG	44.5	23 ± 1	Fluorescent Blue colonies
Fecal Coliforms	m-TEC	44.5	15 min	Colonies which turn yellow after transfer from m-TEC-MUG to m-TEC
Fecal Streptococci	m-ENT	35.0	48	-maroon, red or pink colonies

[a] See appendix

2. - Oxidase Production

A 1% solution of N, N, N, N- tetramethyl -p-phenylene diamine (Appendix) was used to detect the presence of oxidase. The solution was impregnated on clean Whatman No. 5 filter paper discs and allowed to dry. Cultures were taken from heart infusion agar plates with sterile toothpicks and rubbed into the treated filter disc.

Cultures possessing oxidase produce a purple colour on the filter paper, while the negative cultures remained colourless. Results were recorded within two minutes, Pseudomonas aeruginosa is oxidase positive.

3. - Acetamide Reaction

Each Pseudomonas isolate was streaked onto acetamide agar slants (Appendix) and incubated for 24 to 36 hours at 37° C. The production of a dark pink (purple) coloration of the slant by Pseudomonas aeruginosa was regarded a positive result.

4. - Skim Milk Reaction: (Brown and Foster, 1970)

Skim Milk agar (Appendix) was used to determine four reactions; pigment production, casein digestion, fluorescence and grape-like odour by Pseudomonas aeruginosa. Pigment was read as green, yellow or brown. Casein digestion was evidenced by production of a clear zone in the agar around each isolated colony. Cultures were also tested for their ability to fluoresce under a Wood's lamp. Pseudomonas aeruginosa cultures were found to digest casein, produce a grape-like aroma, have a green-yellow pigment, and fluoresce blue-green under UV light.

3.5 - Serotyping

Serotyping Kit (Difco number 3081-32) was used to serotype Pseudomonas aeruginosa. Before serotyping, the diluted antisera was tested against its specific antigen (Difco number 3082-32) for positive control, also it was tested against rabbit serum for negative control.

A day before serotyping each pure Pseudomonas aeruginosa was streaked out on nutrient agar and incubated at 37° C for 18 to 24 hours.

The day of serotyping, the culture was swabbed with a sterile swab, and suspended in 0.85% saline. The suspension was autoclaved for 30 minutes at 121° C, followed by centrifugation at 1000-2000 rpm for 10 minutes. The supernatant was discarded and the bacterial mass was resuspended in 0.75 ml Merthiolate saline (1:10000 Merthiolate in 0.85% NaCl solution).

This constitutes the stable heated antigen and it was tested against each antisera on slide agglutination.

3.6 Restriction Enzyme Analysis of Pseudomonas aeruginosa

Total cellular DNA was extracted as follows: (Bradbury et al, 1984; Bradbury et al, 1985).

A 1.5ml volume of an 18h nutrient broth culture inoculated with Pseudomonas aeruginosa was transferred into a 1.5ml Eppendorff tube and centrifuged in a Microfuge 12, Beckman for three minutes at 7500 x g. The supernatant was discarded and the pellet loosened by vortexing. A 291 ul volume of PEB I buffer (Appendix) containing 10mg/ml lysozyme was added and the mixture incubated for 20 minutes at 35° C. A 9ul volume of 5M NaCl was added and mixed well. A 150ul volume of 10% SDS (Appendix) was added. The solution was mixed gently and incubated for ten minutes at 37° C. After 450 μl volume of 25:24:1:phenol: chloroform:Isoamyl was added to the tube, the mixture was vortexed and centrifuged for six minutes at room temperature at 7500 x g. The upper aqueous phase was removed with a pasteur pipet and transferred into a 1.5ml Eppendorf tube. One ml of 95% cold ethanol was added, the tubes vigorously shaken and stored at -20° C overnight. The mixture was centrifuged for three minutes at 12000 x g, the supernatant discarded and the pellet redissolved in 250 ml DNA wash buffer (Appendix). A total of 500 ul of 95% cold ethanol was added, the mixture stored at -20° C for 20 minutes, and centrifuged at 1200 x g for three minutes. The supernatant was discarded and the pellet allowed to dry at 37° C for 10 minutes. The pellet was dissolved in 100 ul of distilled water and stored at 4° C until digested.

Restriction digests were performed using Sma I according to the manufacturer's instructions (Boehringer Mannheim). A 10 ul aliquot of 2x Sma I buffer was delivered to an Eppendorf tube and 10 ul of extracted DNA was added. A 2 ul sample of Sma I enzyme was added to the mixture and incubated for one hour at 37° C for complete digestion to occur. A one ml volume of 0.15M CDTA + 0.4 mg/ml RNase A was added and incubated for 20 minutes at 37° C. A five ul volume of 5x sample buffer was added to the restriction digest. Samples were electrophoresed on 0.7% agarose gel for 16 hours at 27 volts. Gels were stained with one mg/ml Ethidium bromide in 1 x TAE (Tris base, 1.0 sodium acetate, 0.1 M disodium EDTA) for one hour and destained for 3 hours in distilled water. Photography was done using UV light at 300nm and a red No.23A polaroid 665p/N film with an exposure time of 30 seconds.

Results

4.1 Enumeration of Fecal Indicator Bacteria in Sewers

Fecal coliform, E. coli, fecal Streptococci and Pseudomonas aeruginosa density were performed on all samples from storm and sanitary sewers, using the membrane filtration technique and media described in Table 1.

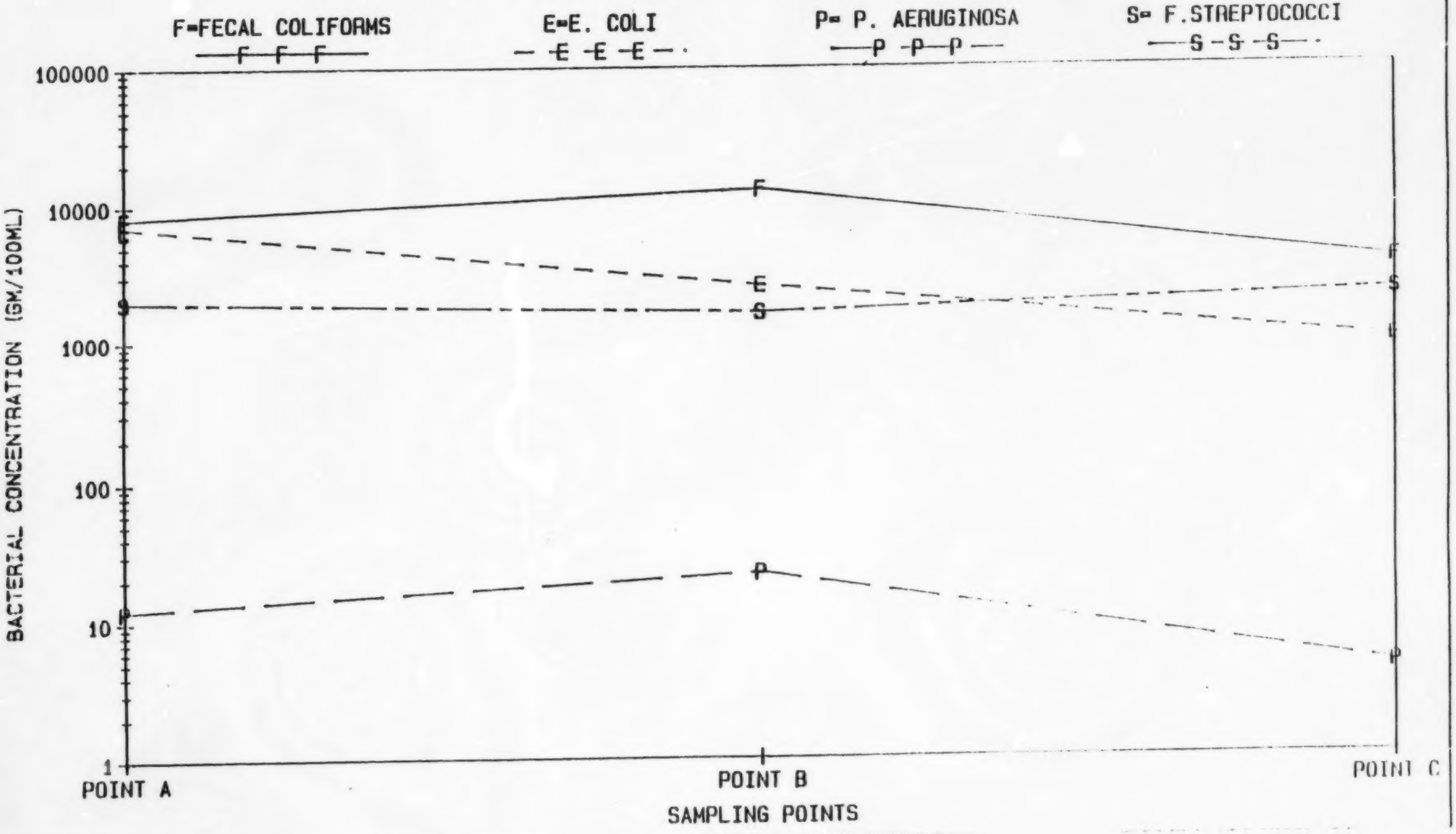
4.1.1 Storm Sewer

- Fecal coliform count in storm sewer varied from 10^3 - 10^4 coliform per 100ml (Fig. 1).
- E. coli counts were in the 10^3 E. coli per 100ml range (Fig. 1).
- The level of streptococci was relatively consistent at three points.
- Pseudomonas aeruginosa levels were low compared to other bacterial indicators. Storm sewers should not contain any human fecal input, and therefore should have low numbers of Pseudomonas aeruginosa organisms.

Geldreich and Kenner (1969) proposed that the sources of fecal bacterial pollution may be differentiated by using a fecal coliform to fecal streptococci FC/FS ratio. It was suggested that if FC/FS ratio is greater than 4.0, the source of pollution is likely of human origin; while if the ratio is less than 0.7 then the source is probably of non-human origin, FC/FS ratios between 0.7 and 4.0 have been considered as intermediate mixed. FC/FS ratios must be employed with some degree of caution as it is time dependent; once discharged into receiving waters, the differential dieoff rates of these organisms and diverse environmental factors may alter their interrelationship to such an extent as to render the FC/FS ratio of limited or no significance in determining the source of bacterial contamination.

Fig. F-1 GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, E. COLI PSEUDOMONAS AERUGINOSA AND FECAL STREPTOCOCCI IN STORM SEWAGE

SAMPLING DATES OCTOBER 21, 22, 28 AND NOVEMBER 18



The three sites contained very high counts of fecal coliforms. Usually, storm sewers contain little fecal contamination, however at this time it is not known what kind of contamination is contributing to the counts observed.

4.1.1 Sanitary Sewer

Sanitary sewers usually contain human fecal waste material which is treated before being deposited in water bodies.

Fecal coliform, E. coli, fecal streptococci concentrations were almost 10 fold more than the levels observed in storm sewers (Fig. 2).

The Pseudomonas aeruginosa count was observed to be around 10000 per 100ml in the sanitary sewers. This was considerably higher than the counts observed in storm sewers.

4.2 Serotyping

In total 285 isolates were serotyped; 148 isolates from storm sewers and 137 isolates from sanitary sewers. The distribution of Pseudomonas aeruginosa serotypes in storm and sanitary sewage varied (Fig. 3 and Table 2).

In storm sewers (Table 2), the most prevalent serotype was 0:6, about 72.3%; followed by serotypes 0:1(15.5%), 0:16(5.4%), 0:11(4.7%), 0:8(1.1%) and 0:12(0.7%).

Sanitary sewers also had serotype 0:6 as the predominant serotype (56.2%). Other serotypes observed from sanitary sewers were serotype 0:10(21.9%), 0:11(13.1%), 0:1(4.4%) and serotype 0:2, 0:4, 0:5, 0:7 were present in very low levels (1.5%).

Serotype 0:16 only appeared in storm sewers, whereas serotype 0:10 was found only in sanitary sewers; however, the percentage recovered was not as high as serotype 0:6.

Fig. F-2 GEOMETRIC MEAN CONCENTRATIONS OF FECAL COLIFORMS, E. COLI PSEUDOMONAS AERUGINOSA AND FECAL STREPTOCOCCI IN SANITARY SEWAGE

SAMPLING DATES OCTOBER 21, 22, 28 AND NOVEMBER 18

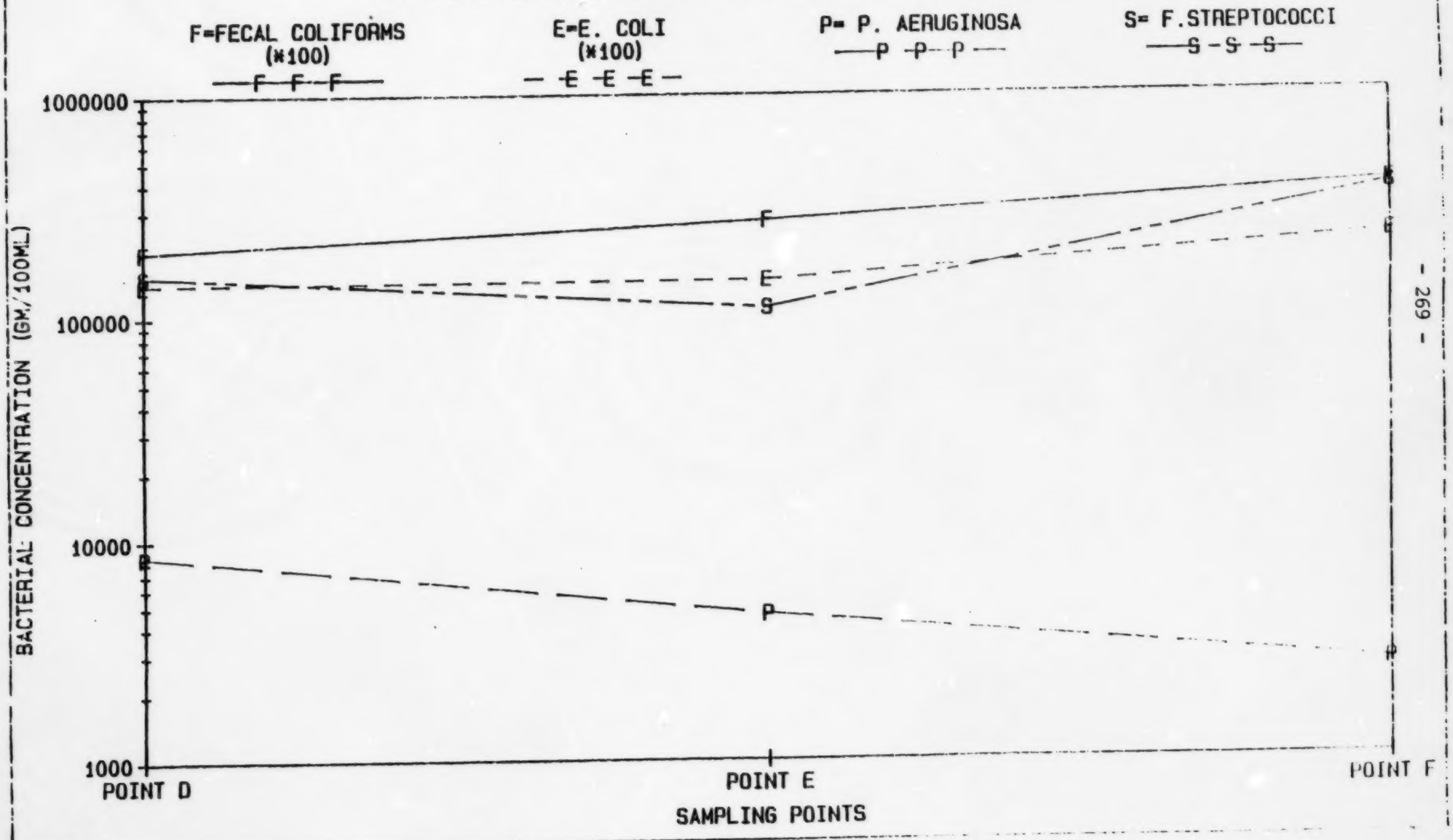


Fig. F-3

PERCENTAGE SEROTYPES OF PSEUDOMONAS AERUGINOSA IN STORM AND SANITARY SEWAGE

SAMPLING DATES - OCTOBER 21 AND 22 AND NOVEMBER 18

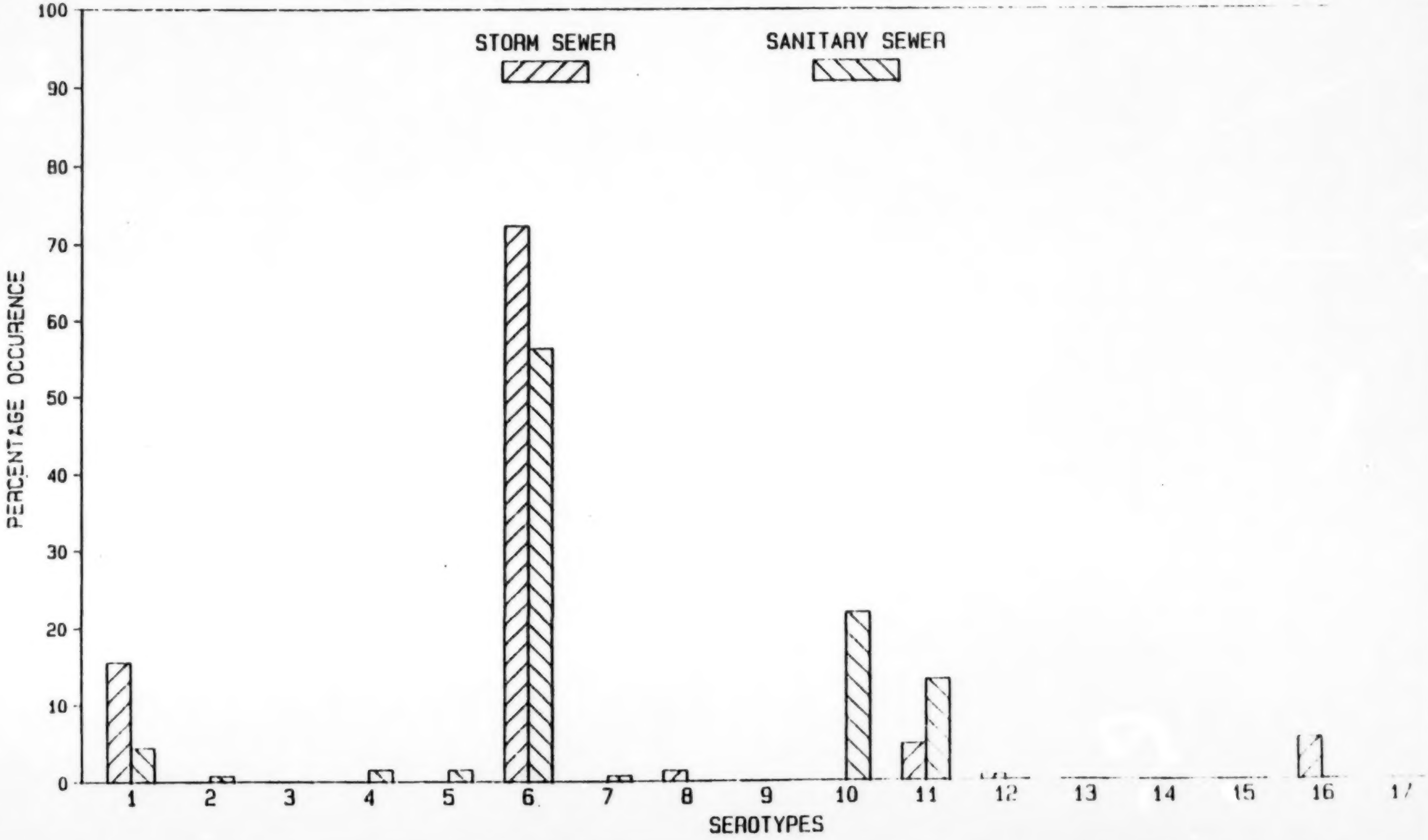


Table F-2

SEROTYPE DISTRIBUTION OF PSEUDOMONAS AERUGINOSA IN STORM AND SANITARY SEWAGE

Total number of isolates: 285
Storm sewer : 148
Sanitary sewer : 137

	Number of isolates serotyped			
Serotype	Storm	%	Sanitary	%
0:1	23	15.5	6	4.4
0:2	0	0.0	1	0.7
0:3	0	0.0	0	0.0
0:4	0	0.0	2	1.5
0:5	0	0.0	2	1.5
0:6	107	72.3	77	56.2
0:7	0	0.0	1	0.7
0:8	2	1.4	0	0.0
0:9	0	0.0	0	0.0
0:10	0	0.0	30	21.9
0:11	7	4.7	18	13.1
0:12	1	0.7	0	0.0
0:13	0	0.0	0	0.0
0:14	0	0.0	0	0.0
0:15	0	0.0	0	0.0
0:16	8	5.4	0	0.0
0:17	0	0.0	0	0.0

4.3 Genotyping

Genotyping was carried out according to the Bradbury et al. 1984, Bradbury et al. 1985 method. Isolates randomly chosen with different serotypes were subjected to restriction enzyme analysis.

Figure 4:

Lane 1 contained lambda bacteriophage marker digested with Hind III.

Lane 2 - 8 contained isolates from storm sewers with serotype 0:6.

Lane 9 and 10 contained isolates from sanitary sewers with serotype 0:6.

Examining Figure 4, it is possible that the same serotype from storm or sanitary sewers have different genotypes (restriction pattern).

Lanes 2, 3, 4, 7 and 8 have the same restriction pattern whereas lanes 5 and 6 have different banding patterns from the previous ones.

Lanes 9 and 10 are from sanitary sewers, serotype 0:6, but each has a different restriction pattern compared to the other isolates.

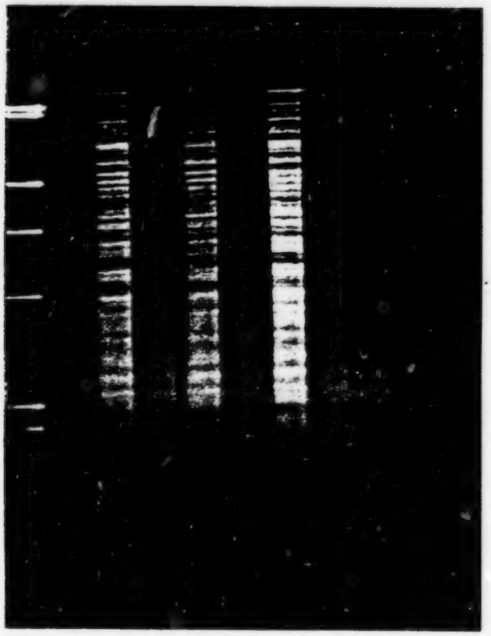
Four different patterns with genotype 0:6 were observed (Fig. 4, Table 3): Two different patterns from storm and two different patterns from sanitary sewers.

Figure 5:

Lanes 2 to 6 contain isolates from sanitary sewers with serotype 0:6.

Lanes 7 and 8 contain isolates from sanitary sewers with serotype 0:1.

Lanes 9 and 10 contain isolates from storm sewers with serotype 0:1 Not all isolates with serotype 0:6 had the same genotype (Fig. 5), lanes 2, 3, 5 and 6 were identical to each other, whereas lane 4 had a totally different banding pattern.



Lane	1	2	3	4	5	6	7	8	9	10
Source	λ	s	s	s	s	s	s	s	S	S
Serotype		6	6	6	6	6	6	6	6	6
REA Pattern		A	A	A	B	B	A	A	C	D

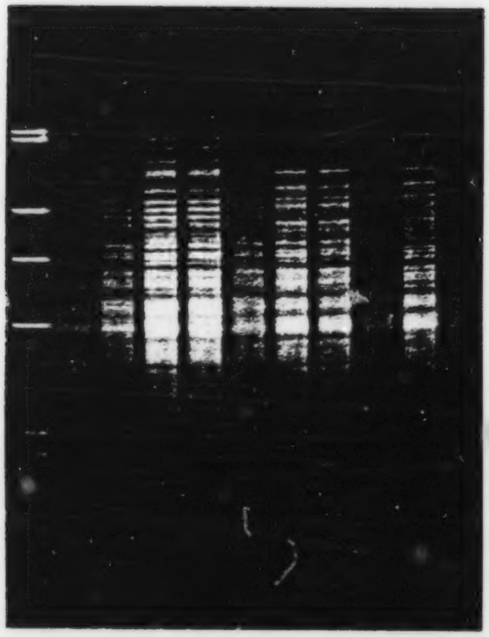
Figure F-4 Agarose (0.7%) gel electrophoresis of total cellular DNA from PSEUDOMONAS AERUGINOSA digested with Sma I endonuclease.

s: storm
S: sanitary

Table F-3

RESULT OF AGAROSE (0.7%) GEL ELECTROPHORESIS OF TOTAL CELLULAR DNA EXTRACTED FROM PSEUDOMONAS AERUGINOSA RANDOMLY:

	ISOLATE NUMBER	SITE	SEROTYPE	LANE	REA PATTERN
FIG 4	382	storm A	6	2	A
	400	storm B	6	3	A
	420	storm B	6	4	A
	1455	storm A	6	7	A
	1490	storm B	6	8	A
FIG 5	1035	sanitary F	6	2	A
	1045	sanitary F	6	3	A
	1540	sanitary E	6	5	A
	1570	sanitary F	6	6	A
FIG 4	915	storm A	6	5	B
	940	storm B	6	6	B
	538	sanitary F	6	9	C
	1505	sanitary D	6	10	D
FIG 5	1528	sanitary D	6	4	E
	1527	sanitary D	1	7	F
	1530	sanitary D	1	8	F
	421	storm C	1	9	G
	436	storm C	1	10	G
FIG 6	361	storm A	16	2	H
	393	storm B	16	3	H
	524	sanitary F	10	4	I
	1504	sanitary D	10	5	J
	526	sanitary F	11	6	K
	413	storm B	11	8	L
	968	storm C	11	9	L
	1052	sanitary F	4	7	M
	432	storm C	8	10	N



Lane	1	2	3	4	5	6	7	8	9	10
Source		S	S	S	S	S	S	S	s	s
Serotype		6	6	6	6	6	1	1	1	1
REA Pattern		A	A	E	A	A	F	F	G	G

Figure F-5

Agarose (0.7%) gel electrophoresis of total cellular DNA from PSEUDOMONAS AERUGINOSA digested with Sma I endonuclease.

s: storm
S: sanitary

Lanes 7 and 8 had the same restriction pattern and both were from sanitary sewers serotype 0:1; however, when these were compared to lane 9 and 10 they displayed a different restriction pattern. Serotype 0:1 from sanitary sewer had a different fingerprint from storm sewer isolates with serotype 0:1.

Comparing Figure 4 to Figure 5, some serotype 0:6 organisms from storm sewer had an identical pattern or serotype (Table 3), to sanitary sewer serotype 0:6. Therefore, in this case, serotype 0:6 from storm sewer had two different genotypes whereas the sanitary sewer had four different genotypes.

Figure 6

Lanes 2 and 3 had isolates from storm sewers with serotype 0:16; both had identical patterns whereas lanes 4 and 5 contained isolates from sanitary sewer serotype 0:10 with totally different genotype each.

Lane 6 contained an isolate from sanitary sewer with serotype 0:11 which had different pattern compared to serotype 0:11 in lanes 8 and 9 from storm sewers.

Lanes 7 and 10 each contained different serotypes, 0:4 from sanitary sewer and 0:8 from storm sewer, they had totally distinct restriction pattern from each other.

DISCUSSION

5.1 Enumeration of Fecal Indicator Bacteria

The result of the study (Fig. 1 and Fig. 2) show that Pseudomonas aeruginosa was found in high concentrations in the human sanitary sewers in relation to high fecal indicator counts. Pseudomonas aeruginosa was also recovered in the storm sewers although in lower concentration than in the sanitary sewer. The concentration in storm sewer of both fecal coliform and E. coli was high especially at point A and approached levels of 10,000 organisms/100ml. FC and EC concentration of this magnitude are suspiciously high for storm water and suggest the presence of sanitary contamination or an extra input from somewhere else. At this point conclusions can not be made about the source of the contamination.

Lane	1	2	3	4	5	6	7	8	9	10
Source		s	s	S	S	S	S	s	s	s
Serotype		16	16	10	10	11	4	11	11	8
REA Pattern		H	H	I	J	K	M	L	L	N

Figure F-6

Agarose (0.7%) gel electrophoresis of total cellular DNA from PSEUDOMONAS AERUGINOSA digested with Sma I endonuclease.

s: storm
S: sanitary

5.2 Serotyping

The concept of serological heterogeneity of Pseudomonas aeruginosa was first recognized in 1912 by Jacob Sthal, who reported that the 55 strains he was investigating were not serologically homogeneous (Verdes and Evans, 1961). The heterogeneous nature of this bacterium was also noted in 1916 by Trommsdorff, who emphasized the importance of distinguishing between the different types of Pseudomonas aeruginosa (Verder & Evans, 19161). These authors failed, however, to recognize and differentiate between the heat stable (0) and heat labile (H) antigenic components of Pseudomonas aeruginosa. The presence of two different antigens was first reported by Brutsaert (1924) and the different antigenic components were investigated specially (Monoz et al. 1945, 1949; Gaby, 1946; Mayr-Harting, 1948; Fox and Lowbury, 1953; Lowbury and Fox, 1954); however, the development of a practical typing scheme, based on the antigens of Pseudomonas aeruginosa, was slow.

Habs in 1957 used boiled antigens and immune sera prepared against these suspensions to devise the first antigenic scheme, suitable for the practical differentiation of Pseudomonas aeruginosa. She reported the differentiation of 70 strains of Pseudomonas aeruginosa into 12 serogroups, based on heat stable 0 agglutinogens. Cross reactions between groups 0:2 and 0:5 were evident and there appeared to be a common antigen among all the strains. Kleinmaier (1957) confirmed Habs' work using slide agglutination with living suspensions of Pseudomonas aeruginosa.

Sandvik (1960) developed an antigenic scheme for this bacterium containing seven 0 groups, into which he could subdivide 87 strains of Pseudomonas aeruginosa of animal origin. When it was compared with Habs' 6/7 serogroups were the same, but the uncommon serogroup was added to the Habs' scheme as 0:13. Vèron (1961) analyzed Habs' cross reacting serogroups 0:2 and

0:5 and subdivided 0:2 into 0:2a and 0:2b and 0:5 into 0:5c and 0:5d. Vèron also emphasized the necessity of cross absorption in order to eliminate the cross reactivity and produce monospecific typing serum thus Vèron described 10 0-groups and 10 H-groups.

Lànyi (1966/67) further extended the work of Verder & Evans (1961); he reported 13 0 types of which 5 would be further divided.

Fisher *et al.* (1969) described a new concept in serotyping of *Pseudomonas aeruginosa*. They differentiated strains of this bacterium on the basis of protective antigens and reported on immunotypes.

Homma *et al.* (1970) developed a typing scheme, based on her 0-serotypes, tested by tube agglutination.

Bergan (1973) compared the typing sets of Habs, Lànyi and Sandvik and reported as the others had, that they were similar. Since then an international panel under the auspices of the subcommittee on *Pseudomonas aeruginosa* and related organisms came up with 17 different serotypes.

Types 1 -12	were prepared using Habs' culture
Type 13	with Veron 0:13 (Sandvik's type 11)
Type 14	with Verder and Evans' 5
Type 15	with Lànyi's 12
Type 16	with Homma's 13
Type 17	with Meitert's type X strain

Table 4 shows the correlation of various serogroups typing schemes based on 0 antigens up to 1969.

Serotyping of *Pseudomonas aeruginosa* was carried out using "*Pseudomonas Antisera*" Kit (Difco) which utilizes the heat stable 0-antigen. Of the 285 isolates serotyped, 148 were isolated from storm sewers, and the remaining 137 from sanitary sewers.

Table F-4

Correlation of Various Serogroup Typing Schemes Based on O Antigens

Habs (1957)	Sandvik (1960)	Verder and Evans (1961)	Lányi (1966/67)	Fisher et al (1969)
1	VII	IV	6	4
2		I	3c	3
3	III	VI	1	
4	IV		11	
5		X	3	7
6	I	II	4	1
7			5abc 5abd	6
8		VIII	5ad	
9	V	IX	10	
10			2	5
11	VI	III	7	2
12		VII	13	
13	II	V		

Table 2 lists the distribution of serotypes of Pseudomonas aeruginosa in storm and sanitary sewage. Serotype 0:6 was the most frequently identified serotype in storm and sanitary sewers, 72.3% and 56.2% respectively. Lànyi (1966, 1967) has also reported that the same serotype was the most common serotype isolated from sewage and surface waters.

Serotypes 0:1 and 0:11 were commonly isolated from storm and sanitary sewers (Table 2). These serotypes were also frequently encountered in studies by Muraschi and coworkers (1966), Lànyi (1966/67); Table 5 shows worldwide frequency.

Serotype 0:16 was only recovered from storm sewers, with a frequency of 5.4% and serotype 0:10 recovered from sanitary sewers 21.9%. This suggests that serotypes 0:16 and 0:10 each belong to a specific source. In theory, storm sewers should only have direct run off from streets, domestic animals, and birds' fecal contamination and sanitary sewers should have human waste inputs. Therefore, since type 0:10 was found in a high frequency in sanitary sewers, and was absent from storm sewers, this serotype may be specific and indicate human fecal contamination. However, at the present time it is not known if serotype 0:10 is specific to humans. Sampling of fecal material from domestic and wild animals should be performed in order to determine if serotype 0:10 is found in animal wastes and if so, at what frequency?

If serotype 0;10 was found to be specific to human feces, water samples of storm sewers taken from various points could be performed and tested for the presence of Pseudomonas aeruginosa serotype 0:10. If the sample was positive for serotype 10, then the sampling site could be examined and the source of the illegal connection determined.

Serotyping by itself does not differentiate within a specific serotype. Even though serotypes 0:1, 0:6 and 0:11 were common to both storm and sanitary

Table F-5

Worldwide Frequency of Incidence of Serotypes of *Pseudomonas aeruginosa*

Investigator	Number of Strains Examined	Source of Strain		Serotype Incidence*	
		Geographic	Host	In Order of Frequency	Per cent Represented
Habs (1957)	70	Germany	Principally Human	6,5,1,3,	70
Sandvik (1960)	87	Norway	Mostly Animal (Bovine)	6,3,13	80
Véron (1961)	142	France, Southeast Asia	Majority Human	5,6,1,8,	60
Verder and Evans (1961)	326	United States	Human and Animal	2,6,11,1	80
Wahba (1965)	1899	Britain and 12 other countries	Milk, water Human, animal	6,2,1,5,	60

* Habs' Classification

sewers, this does not mean that they are identical. To ascertain if this was the case, restriction enzyme analysis was performed on serotype groups in order to determine if identical genotype patterns are present within each serotype group.

5.3 Genotyping (REA)

Initially REA was performed on three isolates using six different restriction enzymes. This step was used to determine which restriction enzyme cut the genomic DNA and produced the best banding patterns on polyacrylamide gels. Figure 7 displays the different banding patterns that were obtained. Sma I produced the best pattern because high molecular weights of DNA bands were distinct and separate unlike the bands produced by Hind III, Bam HI, Eco RI, KPN I and Xho I. Furthermore Sma I is six base cutter cuts at GGGCCC points since *Pseudomonas aeruginosa* has high G/C content of 67%. This indicates that the higher G/C content of *Pseudomonas aeruginosa* may make it more susceptible to cutting by Sma I.

Twenty-seven isolates of *Pseudomonas aeruginosa* were chosen from storm and sanitary sewage and genotyped. Isolates were chosen randomly according to their serotype.

Figure 4 displays the genotypes of sewer isolates from storm sewers, serotype 0:6 and two isolates from sanitary sewers, serotype 0:6. The banding pattern of the storm sewer isolates Figure 4 lanes 5 and 6 were not consistent with others. Isolates in lanes, 2, 3, 4 and 5 are identical where those in lanes 9 and 10 differ. In comparison, five isolates from sanitary sewers, serotype 0:6 Figure 5, lanes 2 -6 did not display the same genotype pattern. Isolates in lanes 2, 3, 5 and 6 were identical, while the isolate in lane 4 was different.

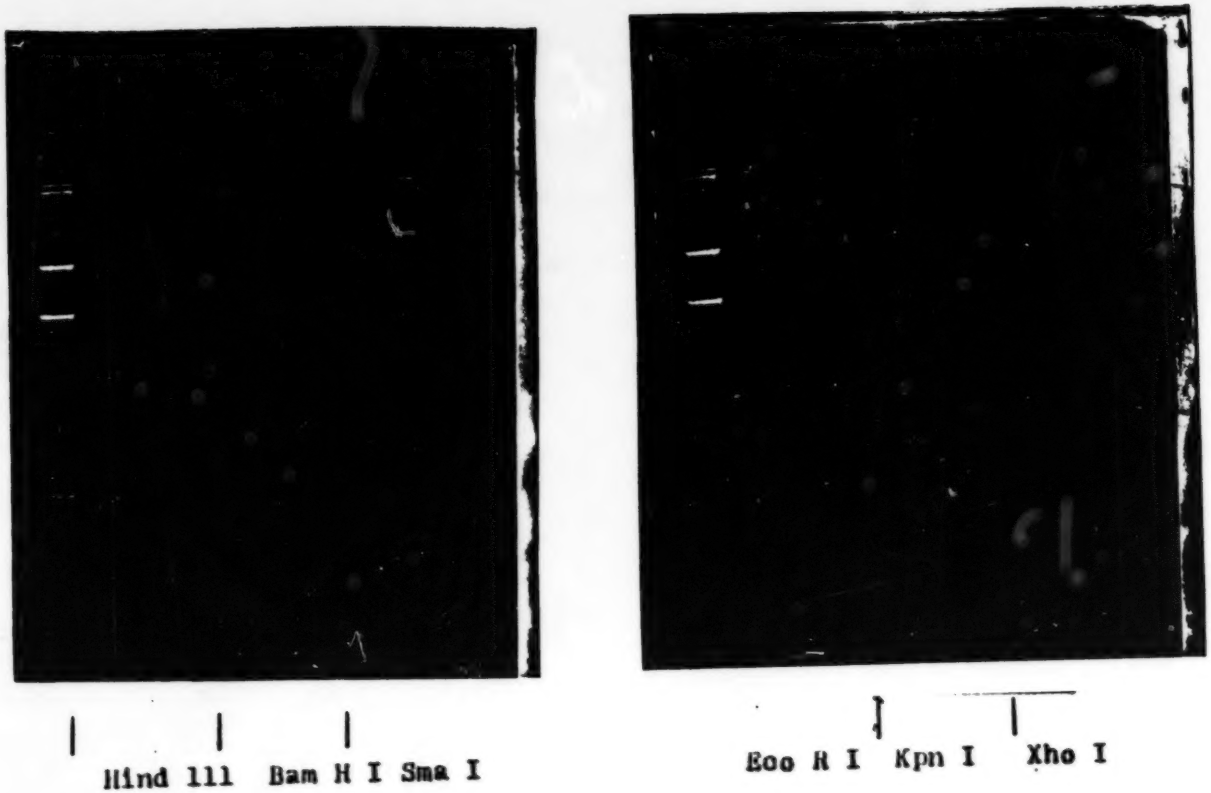

Hind 111
Bam H I
Sma I
Eco R I
Kpn I
Xho I

Figure F-7 Agarose (0.7%) gel electrophoresis of total cellular DNA from PSEUDOMONAS AERUGINOSA DIGESTED with six different endonucleases.

Identical genotype patterns were observed using serotype 0:16 (Figure 6 lanes 2 and 3). Serotype 0:1 from storm Figure 5, lanes 9 and 10 were identical whereas serotype 0:1 from sanitary Figure 5 lanes 7 and 8 were identical but different from storm.

Different genotype patterns were observed within serotype 0:11 from storm sewer Figure 6 lanes 8 and 9 and serotype 0;10 Figure 6 lanes 4 and 5. Differences within the specific genotype were noted in the high molecular weight positions, usually at MW 23.13 K daltons.

Differences were also noted when comparing serotype 0:6 sanitary Figure 4 lanes 9 and 10, and serotype 0:6 storm Figure 4 lanes 2 - 8.

Comparing Figure 4 and Figure 5, it is possible to conclude that some serotypes 6 from storm and sanitary sewer are identical (i.e. they have the same genotype Table 3), but at this time one cannot make any conclusion about the presence and the source of this genotype in storm sewers.

The differences that were seen in REA were not observed using the serotyping technique. For example, not all serotype 0:6 isolates possessed the same genome pattern. This may be accounted for by the fact that the genes responsible for serotyping the 0-Ag comprise a small percentage of the total genome. Therefore, when comparing total chromosomal DNA other genes besides those that code for the 0-Ag, which is characteristic of serotype differentiation, will appear. Thus, the technique of REA proves to be highly specific, in that it further subdivides one serotype into a branch of different genotypes which can then be used in tracing bacterial contaminants from sanitary sewers into storm sewers.

It should be noted that genotypes of Pseudomonas aeruginosa isolates have not been reported by other researchers.

Serotyping, used by itself, was insufficiently discriminatory and of little epidemiological value as some serotypes, namely 0:1, 0:6 and 0:11 were commonly encountered. Conversely, genotyping produced highly specific bands and differences within strains of Pseudomonas aeruginosa were readily demonstrated.

It has been previously suggested that serotype 0:10 was evident only in sanitary sewers. In addition, the genotype banding patterns were observed to be different (Figure 5 lanes 4 and 5). If serotype 0:10 was found in low frequencies in other animal groups, REA may provide highly discriminatory genotypes specific to humans that can be used in tracing the source of input of Pseudomonas aeruginosa in storm sewers from a specific site.

Future studies using a probe developed from either total chromosomal DNA or a specific fragment common to human strains will allow us to hybridize against all other strains.

This information would prove valuable in differentiating between human and non-human fecal waste.

CONCLUSION

Some common serotypes of Pseudomonas aeruginosa are present in both sanitary and storm sewers. The most frequently occurring serotype was 0:6.

Serotype 0:10 appears to exist only in sanitary sewers whereas serotype 0:16 was found only in storm sewage. However no definite conclusion can be made at this time as to the origin of these serotypes (i.e. sanitary or non-sanitary contamination) without further study of the Pseudomonas aeruginosa serotypes found in human and animal feces and those recovered from non-fecally polluted environments.

Genotyping is more discriminative than serotyping, since it can highlight both differences and similarities within a given serotype.

This suggests that REA could be used to specially identify strains from sanitary wastes in storm sewage.

APPENDIX

7.1 Buffers and Solutions

1. Sodium Thiosulphate/EDTA:

$Na_2S_2O_3 - 5H_2O$	3.3g
EDTA	37.2g
Distilled water (dH_2O)	100.0ml

Stir ingredients to dissolve. Add 0.3ml of buffer to each 120ml sample bottle. Autoclave 20 min. at 121° C (15 lbs. pressure).

2. Phosphate Solution:

(A) Dissolve 34.0g KH_2PO_4 in 500ml dH_2O.
Adjust pH to 7.2.
Dilute to 1 liter with dH_2O.

(B) Dissolve 50g $MgSO_4 \cdot 7H_2O$ in 1L dH_2O.

Autoclave both solutions separately for 15 minutes at 121° C. Cool and store at 4° C for up to 1 month. Add 1.25ml of (A) and 5ml of (B) to 1L of dH_2O. Dispense as dilution blanks or for rinse water. Autoclave for 20 minutes at 121° C. (15 lbs. pressure).

3. Saline (0.85%)

Add 8.5g of NaCl per litre of dH_2O. Dispense 5ml in each test tube. Autoclave 15 minutes at 121° C. Store at 4° C.

4. PEB I

SOmM Glucose, 10mM (EDTA), 25mM Tris HCL (PH 8.0)
10mg/ml lysozyme

5. DNA Wash Buffer

0.1 M sodium acetate, 50mM MOPS PH(8.0)

6. 5 x Sample Buffer

1.0ml of 50 x TAE
2.5ml of 1% Bromophenol Blue in 50% ethanol

7. 50 x TAE

302.5g Tris base, 136.08g sodium acetate
tri-hydrate (or 82.03g of anhidrous)
37.5g $Na_2EDTA \cdot 2H_2O$

7.2 Growth Media

The following media (Difco) was prepared according to manufacturer's recommendation:

1. Nutrient agar 1.5%
2. Nutrient broth
3. BHI agar
4. Skim Milk Agar:

Skim milk power	100g
Agar	15g
dH_2O	1L

Add skim milk powder to 500ml dH_2O. Stir without heat for 30 minutes. Add agar to 500ml dH_2 and heat to dissolve.

Autoclave solutions separately for 12 minutes at 121° C. Cool solutions to 55° C. Add milk to agar solution aseptically. Mix thoroughly and dispense into plates. Final pH 6.4 +/- 0.2 . Store at 4H C.

5. **Medium for the Isolation of Thermo Tolerant *E. coli***

(m-TEC - Mug agar):

Proteose peptone No.3	10.0g
Yeast extract	4.0g
Lactose	5.0g
NaCl	7.5g
KH_2HPO_4	3.3g
KH_2PO_4	1.0g
Sodium Lauryl Sulphate	0.2g
Sodium deoxycholate	0.1g
Agar	15.0g
MUG	0.05g
(4-methylumbelliferyl-B-D-glucuronide)	
dH_2O	1L

Mix above ingredients except MUG to 1L dH_2O and heat to 90° C to dissolve. Add MUG just before autoclaving. Autoclave for 15 minutes at 121° C. Cool to 55-60° C and dispense in sterile square petri dishes. Final pH 7.1 +/- 0.1. Store at 4° C.

6. M-Enterococcus Agar (mENT):

M-Enterococcus agar (Difco)	42g
Sterile dH_2O	1L

Weight out agar in a sterile beaker using an alcohol flamed spatula. Heat to dissolve agar (93° C). Cool and dispense into square petri dishes. Final pH 7.2 +/- 0.2.

7. Medium for *Pseudomonas aeruginosa* (m-PA):

L-lysine monohydrochoride	5.0g
Yeast extract	2.0g
Xylose	2.5g
Sodium thiosulfate	5.0g
Magnesium Sulphate, anhydrous	1.5g
Sucrose	1.25g
Lactose	1.25g
Sodium Chloride	5.0g
Ferri ammonium citrate	0.80g
Sodium disoxycholate	0.10g
Phenol red	0.08g
dH_2O sterile	800ml

Mix above ingredients and adjust pH to 7.6. Add 15g agar. Heat to 93° C to dissolve agar. Cool to 60° C and stir in Antibiotic solution*. Dispense into square sterile petri dishes. Store at 4° C after solidification. Final pH 7.1 +/- 0.1.

* Antibiotic solution:

A) Sulfapyridine	0.1760g
B) Kanamycin sulphate	0.0085g
C) Naladixic acid	0.0370g
D) Cycloheximide	0.1500g

Dissolve A-D in 200ml sterile dH_2O. Heat to 50° C to dissolve antibiotics.

8. Acetamide Agar Slants

NaCl	5g
K_2HPO_4	1.4g
KH_2PO_4	0.7g
Acetamide	10.0g
Mg SO_4-7 H_2O	1.0g
Phenol red	0.012g
Agar	15.0g
dH_2O	1L

Add all ingredients except agar. Adjust pH to 6.8 then add agar. Heat to 92° C, autoclave for 15 minutes at 121° C. Final pH 6.8 +/- 0.2. Dispense into sterile screw cap test tubes.

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APPENDIX - G

BIFIDOBACTERIUM SP.

Bifidobacteria are gram-positive organisms which may have club-shaped, bifid ends. Colonies are about 1mm in diameter, smooth, entire, convex, opaque, glistening and white. Bifidobacteria found in humans include B. adolescentis, B. catenulatum, B. breve, B. longum, B. globosum and B. infantis. Even though they have been isolated, their significance has not been determined because biochemical fermentation reactions, and microscopic examination do not definitely differentiate between all Bifidobacterium species. Polyacrylamide gel electrophoresis of cellular proteins or other genetic investigations, which can give conclusive identification, can be time-consuming.

The only pathogenic Bifidobacterium sp. is B. dentium which is found in the mouth, intestines, and in mixed infections of the lower respiratory tract. Because it is an obligate anaerobe, it produces acetic and lactic acids in peptone-yeast-extract-glucose broth and ferments many sugars. According to GLC results, bifidobacteria produce more acetic in comparison with lactic acid than other gram-positive, non-sporeforming anaerobic bacilli in humans.

Bifidobacteria recovery from membrane filtration is hampered by the growth of other bacteria on the media. In YN17 (Mara and Oragui, 1983), a media used for bifidobacteria cultivation, the antibiotics present prevent growth of other bacteria facilitating isolation and making identification easier. However, the addition of actidione (cycloheximide) to inhibit mold growth, and the addition of specific vitamins, minerals or other antibiotics, have been considered to fortify the media.

Bifidobacteria have the ability to alter their appearance and this makes their classification difficult.

Method of Recovering Isolates

Bifidobacteria isolates were obtained by diluting a sample and passing it through a membrane filter.

Media

On YN17 (blue), bifidobacteria appears as very dark blue to black colonies. The green-tinged colonies are usually streptococci. Other media used included MRS (de Man et al, 1960) by Gibco or Oxoid. It was suggested that by the addition of cysteine hydrochloride, a reducing agent, the media becomes more anaerobic - thus helping to make the environment more conducive to this organism's growth requirements. YN17, without antibiotics or indicator, and TPY (recommended on Page 1423, Bergeys Manual, gram-positive organisms) were also used as growth media. Bifidobacteria responded to culture transfers and environmental stress by changing their cell morphology and biochemical reactions.

Method of Analysis

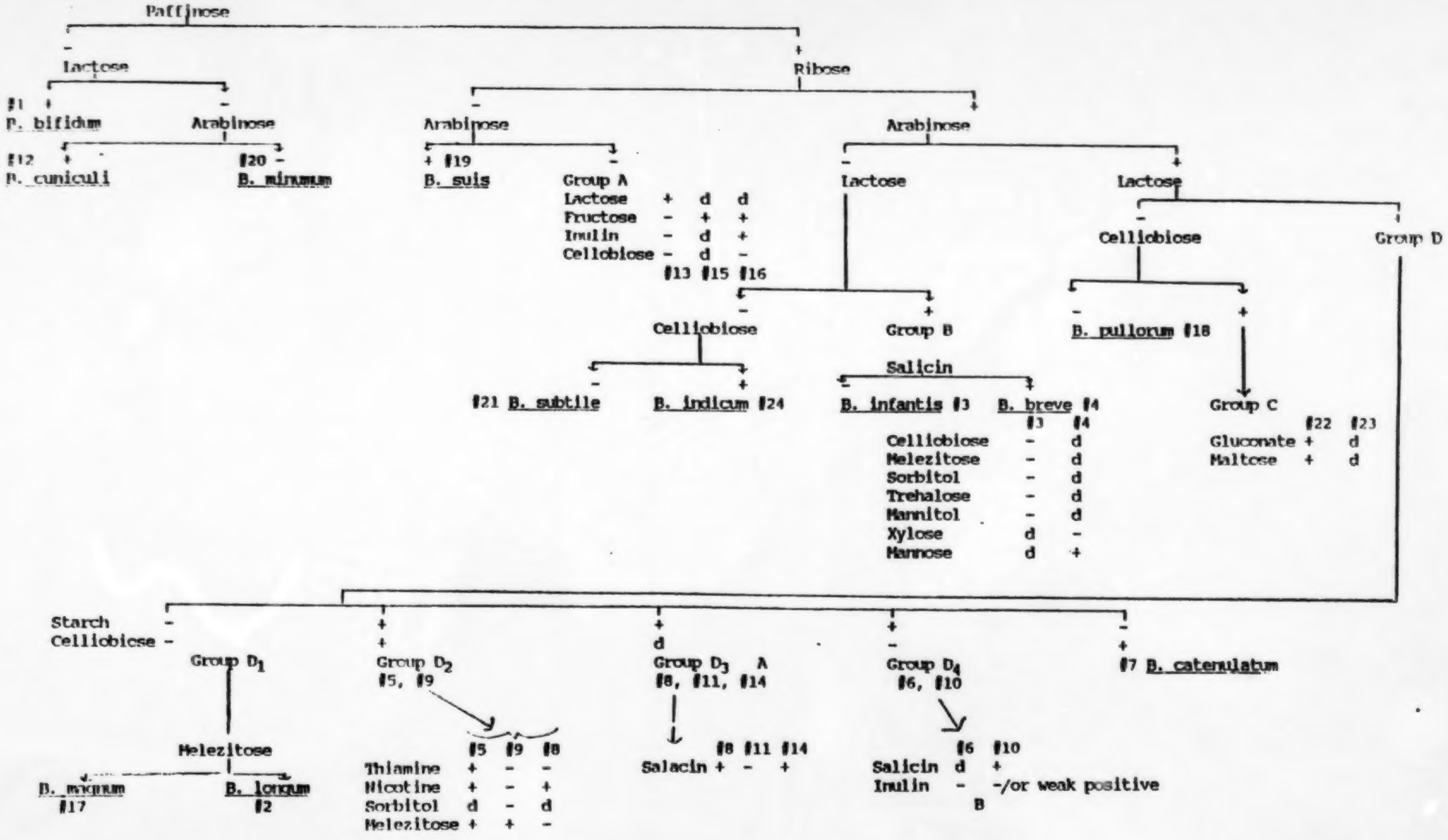
The sample, either pure or from sewage, was diluted and tested by the MF procedure (i.e. sewage) or by spread plating (i.e. animal sources).

The plates were incubated anaerobically for 48 hours at 37° C using BBL anaerobic Gas Pack anaerobic systems (70304) and indicator strips (70504) in an anaerobic jar.

Dark blue (YN17) colonies and circular, mucoid, white translucent colonies (TPY, MRS, YN17) were chosen for further examination. All information concerning growth, source, etc. was recorded. The colonies were labelled and inoculated into 5 ml MRS broth with cysteine hydrochloride added just before use. These tubes were incubated for 48 hours at 37° C anaerobically. From the tubes, isolated colonies were streaked on different media. The plates were incubated anaerobically for 2 days. Each isolate was streaked out on plates once more to ensure a pure culture. The culture was tested with Gram reagent (3% potassium hydroxide) catalase reagent (3% hydrogen peroxide) and a slide was made of the organism using Crystal Violet. (*Bifidobacterium* is gram-positive; the stain was used to observe cell appearance).

The following tests were applied for *Bifidobacterium* characterization: Gram stain, catalase, gelatin, bile esculin, Kligler's iron agar, sorbitol, xylose, cellobiose, melezitose, arabinose, lactose, mannitol, melibiose, ribose, mannose, trehalose, raffinose, fructose and litmus milk (see Appendix). All inoculated tests were incubated at 37° C in an anaerobic jar. The sugars were examined after 24 and 48 hours. A negative control for all the sugar reactions was incubated along with the other tests. The sugar tubes were discarded after 2 days. Other results were conclusive within 5-7 days. The appended flow chart was devised by Xu Yan, Arif Somani, R. Desjardins to help in the analysis of the results. Please note that numbers on the flow chart correspond to Table 15.51 (Page 1428) Bergeys Manual, gram-positive organisms. Table 5 in the paper by Mitsuoka (1982) is also appended as a useful classification reference.

Identification Flow Chart for Bifidobacterium spp.*


Raffinose
-
Lactose
#1 +
B. bifidum
-
Arabinose
#12 +
B. cuniculi
#20 -
B. minimum
+
Ribose
-
Arabinose
+ #19
B. suis
-
Group A
Lactose + d d
Fructose - + +
Inulin - d +
Cellobiose - d -
#13 #15 #16
+
Arabinose
-
Lactose
-
Celliobiose
-
#21 B. subtile
+
B. indicum #24
+
Group B
Salicin
B. infantis #3
B. breve #4
#3 #4
Celliobiose - d
Melezitose - d
Sorbitol - d
Trehalose - d
Mannitol - d
Xylose d -
Mannose d +
+
Lactose
-
Celliobiose
-
B. pullorum #18
+
Group C
#22 #23
Gluconate + d
Maltose + d
+
Group D
Starch -
Celliobiose -
Group D1
Melezitose
B. magnum
#17
B. longum
#2
+
+
Group D2
#5, #9
#5 #9 #8
Thiamine + - -
Nicotine + - +
Sorbitol d - d
Melezitose + + -
+
d
Group D3 A
#8, #11, #14
#8 #11 #14
Salacin + - +
+
-
Group D4
#6, #10
#6 #10
Salicin d +
Inulin - -/or weak positive
B
-
+
#7 B. catenulatum

* Note: Numbers correspond to Table 15.51, Page 1428, Bergeys Manual, Gram positive organisms.

A: #8 and #14 are very difficult to differentiate with certainty. Perhaps use DNA homology.

B: Some strains from piglets are positive.

Table Characteristics and distribution of species and biovars of bifidobacteria isolated from intestines of humans and animals[a]

Species and biovars			Mono-					Saccharide												Poly-alcohol			Glucoside			
			Pentose			Hexose		Di-						Tri-		Poly-										
	Growth at 46.5 C	Clot of litmus milk	Arabinose	Xylose	Ribose	Mannose	Fructose	Sucrose	Maltose	Cellobiose	Lactose	Trehalose	Melibiose	Raffinose	Melezitose	Dextrin	Starch	Glycogen	Inulin	Mannitol	Sorbitol	Inositol	Esculin	Salicin	Amygdalin	α-Methyl-glucoside
bifidum a	−	+	−	−	−	−	+	+s	−	−	+	−	(+)s/−	−	−	−	−	−	−	−	−	−	−	−	−	−
bifidum b	−	+	−	−	−	−	+	−	−	−	+	−	+/+s	−	−	−	−	−	−	−	−	−	−	−	−	−
infantis																										
ss. *infantis* a	−	+	−	−	+s	+s	+s	+	+	(+)s	+	−/(+)s	+	+	−	−	(+)s	−	+	−	−	−	−	+	−	v
ss. *infantis* b	−	+	−	−	+	+s	+	+	+	−	+	−/(+)s	+	+	−	+s	+s	−	+	−	−	+	−	(+)s	−	v
ss. *liberorum*	−	+	−	+	+	+s	+	+	+	−	+	+s	+	+	−	(+)s	(+)s	−	−	−	−	+	+s	+s	−	+s
ss. *lactentis*	−	+	−	+	+s	+s	+	+	+	−	+	−	+	+	−	−	+s	−	+	+	−	(+)	−	−	−	+s
breve																										
ss. *breve* a	−	+	−	−	+	+	+	+	+	+	+	−	+	+	−	v	v	v	(+)s/−	+	+	−	+	+	+	+
ss. *breve* b	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+s	v	v	v	(+)s/−	+	+	−	+	+	+	+
ss. *breve* c	−	+	−	−	+	+	+	+	+	+	+	−	+	+	+	v	v	v	(+)s/−	+	+	−	+	+	+	+
parvulorum a	−	+	−	−	+	+	+	+	+	+	+	−	+	+	−	+	+	v	v	−	−	−	+	+	+	+
parvulorum b	−	+	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	v	v	−	−	−	+	+	+	+
thermophilum a	+	−	−	−	−	−	+	+	+	v	−	v	+	+	−	+	+	+	+	−	−	−	(+)s	(+)s	(+)s	+s
thermophilum b	+	−	−	−	−	−	+	+	+	+s	−	−	+	+	+	+	+	+	−	−	−	−	(+)s	+s	(+)s	+s
thermophilum c	+	+	−	−	−	−	+	+	+	v	+	+	+	+	−	+	+	+	+	−	−	−	(+)s	+s	(+)s	+s
thermophilum d	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+s	+s	+s	+s
adolescentis a	−	+	+	+	+	+/+s	+	+	+	+s	+	+	+	+	−/+s	+	+	+	v	+	+	−	+	+	+	+
adolescentis b	−	−	+	+	+	+s/+	+	+	+	+s	+	v	+	+	−/+s	+	+	+	v	+	−	−	+	+	+	+
adolescentis c	−	v	+/+s	+/+s	+	+s/+	+	+	+	+s	+	v	+	+	−/+s	v	v	v	v	−	+	−	+	+	+	+
adolescentis d	−	v	+/+s	+/+s	+	+s/+	+	+	+	+s	+	v	+	+	−/+s	+s	+s	−	−	−	−	−	+	+	+	+
longum																										
ss. *longum* a	−	+	+	+	+	+s/−	+	+	+	−	+	−	+	+	+	±	±	−	−	−	−	−	−	−	−	+s
ss. *longum* b	−	+	+	+	+	+	+	+	+	−	+	−	+	+	+	±	±	−	−	−	−	−	−	−	−	+s
animalis a	−	v	+	+	+	−	+/+s	+	+	−	+	−	+	+	−	±	±	−	−	−	−	−	±	±	−	+
animalis b	−	+	+	+	+	+	+/+s	+	+	−	+	−	+	+	−	±	±	−	−	−	−	−	±	±	+s	+s
pseudolongum a	−	+	+	+	+	−	+s	+	+	−/+s	+	−	+	+	−	+	+	+	−	−	−	−	−	±	−	+s
pseudolongum b	−	+	+	+	+	+s	+s	+	+	+/+s	+	−	+	+	−	+	+	+	−	−	−	−	−/+s	+s	−/+s	/+s
pseudolongum c	−	+	+	+	+	+s	+s	+	+	+s	+	−	+	+	+	+	+	+	−	−	−	−	±	±	±	+s
pseudolongum d	−	−	+	+	+	+s	+s	+	+	+s	−	−	+s	+	−	+	+	+	−	−	−	−	−	+	−	+s

[a] Symbols: + =positive; − =negative; (+)=weak positive; v=variable; +s=slowly positive.

APPENDIX - H

FECAL STREPTOCOCCI

The following identification scheme (ASM, 1985) has been prepared by Gary Horsnell, Ministry of the Environment (1987) and has been employed at the Ministry of the Environment for fecal streptococcal identification.

Introduction

The fecal streptococci of interest in this study may be illustrated by the tables given below (Modified by Hartman et al, 1966).

Fecal Streptococci

Enterococci	S. faecalis var faecalis	Group D
	S. faecalis var liquefaciens	
	S. faecalis var zymogenes	
	S. faecium	
	S. faecium var casseliflavus	
	S. durans	
Viridans group	S. bovis	
	S. equinus	
	S. mitis	
	S. salivarius	

S. avium (GRP D, Q)

Media

The fecal streptococcus isolates were picked from recovery media such as m-E agar and m-Enterococcus agar. (Details of preparations can be found in the Appendix under Media.) Colonies of all sizes that appeared pink with a blue halo on m-E and pink to maroon on m-Enterococcus were chosen. Source, ENTEROCOCCI

	Bile esculin	Todd Hewitt 45° C	6.5% NaCl	Arginine	Pyruvate	Arabinose	Mannitol	Sorbose	Gelatin	Haemolysis	Yellow Pigment	Group D
S. faecalis												
V. faecalis	+	+	+	+	+	–	+	–	–	γ/α	-	+
V. liquefaciens	+	+	+	+	+	–	+	–	+	γ/α	-	+
V. zymogenes	+	+	+	+	+	–	+	–	+/–	β	-	+
S. faecium	+	+	+	+	-	+	+	–				
S. faecium												
V. casseliflavus	+	+	+	+/-	-/+	+	+	–	–		+	+
S. durans	+	+	+	+	-	–	-	–	–		-	+
S. avium	+	+/-	+/-	-	+	+	+	+	–		-	+ (also Q)

dilution, media were recorded as well. Care was taken to pick (with a sterile loop or needle) only one colony for each isolate sample.

Method of Collecting Isolates

The colony was streaked onto Brain Heart Infusion (Difco) agar slants and incubated for 18-24 hours at 37° C. The following day, the slants were covered with sterilized paraffin oil for storage. The isolate would no longer grow but would be kept free from contamination.

Method of Identification

Prior to inoculation, the BHI plates were dried under a laminar flow hood for approximately 15 minutes. The plates, labelled according to the isolate number, were streaked in 4 quadrants to obtain isolated colonies and incubated at 37° C for 18-24 hours. The plates were then examined to determine how many different types of colonies were present by looking for a) colour; b) colonial morphology; and c) contamination. Each different type of colony, except the latter, was transferred onto a BHI plate divided into 6 sections to make a reservoir (i.e. a spread or patch of growth). This was done to obtain a larger volume of bacterial growth for further testing.

The next day, wet mounts of each sample were made and examined under the microscope. Streptococci are cocci, ovoid to spherical in shape. They occur in short chains (3-6 cells) on agar or longer chains (more than 6 cells) in broth or in pairs or individually. They do not appear as tetrads. Next, the following tests were performed to separate them from other gram-positive cocci.

Each isolate was tested with 3% H_2O_2 (hydrogen peroxide) for the catalase reaction. The test solution was first tested with known stock culture controls of S. faecium and Pseudomonas aeruginosa to make sure the solution would perform correctly. Both the catalase reagent and Gram reagent (KOH) were kept for 2 weeks. S. faecium is catalase negative; i.e. no reaction when the culture is tested with reagent while P. aeruginosa is catalase positive. Introduction of culture to reagent produces bubbles.

For the Gram test, potassium hydroxide (KOH) was used. The culture was mixed with a drop of KOH for about one minute with a loop. A gram-negative such as P. aeruginosa breaks down the cell wall and the contents disperse. The mixture becomes thicker, syrupy and stringy when you raise the loop from the slide. A gram-positive culture (i.e. Streptococcus) will give no reaction with KOH.

The isolate was tested on rabbit blood agar (see Appendix) with a single horizontal streak. The plates were incubated in an anaerobic jar using BBL anaerobic gas pack anaerobic systems 70304 and indicator strips 70504 for 48 hours at 37° C (To ensure media efficiency, test with a known positive (i.e. S. faecalis v. zymogenes) and negative (S. faecium). A positive hemolysis will give a clearing of the media around the streaked zone.

All results were recorded on bench sheets. Another isolate reservoir was streaked onto BHI. Much care was taken in the streaking process since blood agar and BHI can be easily contaminated.

Streptococci can be divided into four general groups:

a) the viridans group
b) the lactic group
c) the pyogenic group
d) the fecal or enteric group

There are 6 tests used in this method to help to separate the fecal group from the others. These are:

1. Bile esculin
2. growth at 45° C - using Todd Hewitt broth (see Media section in Appendix)
3. growth in 6.5% NaCl
4. arginine

5. haemolysis
6. antigen serotyping (Group D)

To identify an isolate as an enterococcal group D fecal streptococcus only the first four tests plus presence of the Group D antigen are required.

The isolates were also inoculated into pyruvate, mannitol, sorbose, lactose, melibiose, gelatin, litmus milk, arabinose and starch agar.

The following observations and conclusions can be made from the previous table:

a) S. faecium variants are usually arabinose+, pyruvate- and never gelatin+ or haemolytic+. However S. faecium var casseliflavus which is the only yellow pigmented organism in the enterococcal group can be arginine+/-, pyruvate+/-, (and arabinose+).

b) S. faecalis variants are usually always pyruvate+, arabinose- and may or may not be gelatin+, haemolytic+.

It is possible for an organism to lose its ability to produce a positive result (i.e. to ferment a sugar). However, an organism cannot gain an ability to react if it naturally did not have it before, unless the cell obtained a plasmid-carrying gene through interaction with other, similar organisms.

Biochemical Test Results

Test	Positive Reaction	Example/Notes
bile	blackening	
arginine with paraffin oil (anaerobic)	pink	(all Group D are positive) except *S. avium* and sometimes *S. faecium* var *casseliflavus*
6.5% NaCl growth	growth	
arabinose fermentation	yellow	S. faecium
lactose fermentation	yellow	
pyruvate fermentation	yellow	S. faecalis variants
mannitol fermentation	yellow	
sorbose	yellow	S. avium
litmus milk	acid, alkali curds/reduction	
gelatin	liquefaction	S. faecalis liquefaciens S. faecalis zymogenes
Todd Hewitt	growth	
melibiose	yellow	S. faecium
blood	clearing	S. faecalis var zymogenes
starch agar		S. bovis

Serology

By looking at the profile of all the results it was possible to make an identification for each isolate. If, by chance, the first 5 tests produced a negative result, it was necessary to perform a serology test to see if Group D was present.

Group-specific antigens are usually carbohydrate structural components of the cell wall. These antigens can be extracted in soluble form and identified by precipitation with homologous antisera.

There are a number of ways to extract these antigens from the cell wall including:

1. Hot HCl extraction
2. Hot formamide extraction
3. Autoclave extraction
4. Sonication, and
5. Enzyme extraction

Each extraction method has certain advantages and disadvantages. Generally the autoclave extraction method and the enzyme extraction method are simple yet reliable procedures for all groups including Group D. Some Group D streptococcus species (i.e. S. bovis, S. equinus and S. avium), however, contain relatively small amounts of this antigen and these may require more severe extraction procedures (i.e. sonication).

The antigen-antisera precipitation reactions can be performed in various ways including:

1. Capillary precipitin test
2. Slide agglutination reaction
3. Electrophoretic methods.

Probably the simplest method to employ is the slide agglutination procedure whereby group-specific antibody coated latex particles are reacted with the antigen extract.

There are commercially prepared kits available which provide the enzyme for an enzyme extraction, a reaction slide and antibody coated latex particles for various serogroups (generally groups A, B, C, D, F, & G). These latex particles can also be reacted with extract from any other extraction procedure.

The method is as follows:

1. A pure isolate is inoculated into BHI broth and incubated for 18-24 hours at 37° C. (The broth is brought up to 1% glucose by adding 8g/l and put in suitable screw cap tubes for centrifugation.)

2. Next day, the broth culture is centrifuged at 3000 rpm for 10 minutes to pack the cells.

3. The clear liquid broth (supernatant) may be reacted with the group D antisera (Group D antibody coated latex particle suspension) (Streptex Latex Group D suspension Wellcome Diagnostics) to determine the presence of the Group D antigen. A drop of the suspension is placed on a tile to which a drop of supernatant is added. The tile is placed on a Variable Speed Rotator. Observation is made. If agglutination occurs, the reaction is recorded as a Group D positive. If it doesn't take place, the extraction procedure continues.

4. The supernatant is carefully decanted from the tube and put in disinfectant.

5. Five 5mL of physiological saline (0.85% NaCl) is added to the tube to resuspend the cells.

6. The tube is once again centrifuged at 3000 rpm for 10 minutes to pack the cells.

7. The clear saline is decanted into a disinfectant (Dettol).

8. Then 0.4 ml extraction enzyme (Streptex Extraction Enzyme S. griseus Wellcome Diag.) is added to the tube which is shaken to resuspend the cells.

9. The tubes are incubated at 37° C (water bath) for 1 hour.

10. Following this, the tubes are autoclaved for 30 minutes 15 psi (121° C).

11. The tubes were cooled and centrifuged at 3000 rpm for 10 minutes.

12. The clear liquid phase is tested for the presence of the group D antigen. 1 drop clear extraction liquid is added to 1 drop group D latex suspension. A group D positive reaction is indicated by agglutination (granular appearance in mixture). If the mixture remains clear, the isolate is group D negative.

APPENDIX - I

PSEUDOMONAS AERUGINOSA

Introduction

Pseudomonas aeruginosa can be found in soil, water, sewage, plants and the mammalian gut. The organism is pathogenic for humans, plants, insects and some animals. Since nutritional requirements are simple, and the organism can make use of organic compounds, and exist in water of ambient temperature, Pseudomonas aeruginosa is commonly found in hospital wards. Pseudomonas aeruginosa produces many toxins and enzymes.

Since Pseudomonas aeruginosa is an opportunistic pathogen, and has been the cause of several infections carried through contaminated waters to a vulnerable host. Pseudomonas aeruginosa has been shown to be the cause of outer ear infections in swimmers (Seyfried and Fraser, 1978).

A count of 10 organisms/100 ml indicates recent fecal pollution (MOE, 1983).

The medium used for Pseudomonas aeruginosa enumeration is m-PA (Levin and Cabelli) (Appendix). The antibiotics used in the medium and the high incubation temperature, help make the medium selective for Pseudomonas by inhibiting growth of other heterotrophic bacteria.

Method of Sampling

The sample was tested using the membrane-filtration technique and dilutions were made as required.

The m-PA plates were incubated for 48 hours at 41.5 ± 0.5^0 C. The plates were placed, inverted, in a cakette moistened with paper toweling.

Pseudomonas colonies appeared flat, spreading, brownish-green, or tan. Typical colonies were confirmed by streaking them on Skim Milk Agar (Appendix) with incubation at 37° C for 48 hours. A positive result was indicated by a clearing of the media and a fluorescent pigment. The culture was then transferred onto a BHl slant and numbered.

Analysis of Pseudomonas Isolates

The isolates were streaked from the BHl slants onto nutrient agar (Difco) to obtain isolated colonies. These plates were incubated 18-24 hours at 37° C. The next day, the plates were examined to determine whether the plate had only one type of colony. If the organisms on the plate appeared as a pure culture, the colonies were tested with:

a) Gram reagent which was tested against a known positive and negative control. For example, Streptococcus (faecium) (G+) was used as a negative (not sticky) and our Pseudomonas aeruginosa (G-) control was used as a positive (sticky, syrupy).

b) Oxidase reagent. On a filter paper (Whatman #1) which was cut to fit inside a petri dish, the oxidase reagent was applied to the paper and allowed to dry. (If the oxidase appeared blue from the storage bottle, it was discarded). A colony was taken from the plate with a wooden applicator and stamped onto the paper. A positive reaction resulted when the spot turned blue, violet. This occurred a few minutes after being tested. Results were recorded. In addition, the culture was also streaked on acetamide slants and skim milk agar.

The acetamide tubes were incubated at 37° C for 72 hours. A positive appeared as bright pink.

The skim milk agar comprised 4 different tests:

a) A clearing of the medium surrounding the streaked zones (+);

b) The culture appeared to fluoresce under an Ultra Violet light source (short wavelength). These stains produced water-soluble pigments (pyoverdins). Pyoverdin production is dependent on nutritional factors.

c) The growth on the plate appeared as yellow-green to brown (pyocyanin pigment production)+.

The pyocyanin production, which is a blue water-soluble non-fluorescent pigment is excreted by Pseudomonas aeruginosa into the medium.

The greenish appearance is caused by the presence of the (blue) pyocyanin and the yellow pyoverdins.

d) Pseudomonas aeruginosa strains possess a grape-like odour.

Sample Preparation for Serotyping

1. All cultures showing positive results were sub-cultured to reservoirs on Nutrient agar (Difco) with a sterilized swab.

   Incubation of the inverted plates was for 18-24 hours at 37° C.

2. From the reservoirs, thick suspensions of growth were made into 10 mL of sterile 0.85% NaCl (Appendix). The tubes were autoclaved for 30 minutes at 121° C with a slow exhaust.

3. The autoclaved suspension was centrifuged at 1000-2000 rpm for 10 minutes and the supernatant was discarded.

4. The pellet was resuspended in 0.75 mL of a solution consisting of 0.85% NaCl and 1/10,000 merthiolate (Appendix).

Dilution of Sera

The following steps were followed using the 17 sera from the Pseudomonas aeruginosa antisera kit (Difco):

1. Resuspended sera by adding 1 mL sterile distilled water to dissolve contents.

2. Sterilize approximately 20 screw cap tubes.

3. Pipet 0.9 mL of the 0.85% NaCl-merthiolate solution into each tube. (Label tubes 1-17).

4. Using a clean pipet each time, pipet 0.1 mL of sera into the appropriate tube.

Testing the Sera

Every day, before the samples were tested the following procedure was followed. (Both positive and negative controls were tested first).

1. Antiserums 1-17, as already described, were diluted 1:10 (0.1 mL antisera + 0.9 mL NaCl and merthiolate solution).
2. Antigens 1-17, which were already in solution were mixed (0.1 mL antigen and 0.9 mL rabbit sera*. *Rabbit serum was first diluted 1/10).

Positive Control

1. Place a drop of antiserum 1 on tile.
2. Place a drop of antigen 1 on top.
3. Next, put the tile on a Variable Speed Rotator (Yankee 070504, Clay Adams, Parsippany, NJ) so that it will rotate gently. Leave for a few minutes. There should be agglutination. If however, there isn't, use the antigen full strength (not diluted with rabbit serum). Lack of agglutination can occur if there is too much organism present. A prozone is created (meaning an excess of antigen), thus no agglutination occurs.
4. Each antisera should be tested with its corresponding antigen.

Testing Collected Isolates

1. Place a drop of each antisera onto a separate square of the tile.
2. Add the diluted, prepared and mixed sample - one drop per square.
3. Record tile and sample number for referral.
4. Place on the Rotator.
5. Observe closely after a few minutes for agglutination (forming of granules).
6. Record results.

Negative Control

1. A drop of Antiserum 1 is mixed with a drop of antigen 2.

			Result
Antigen 1	+	Antiserum 1	+
Antigen 2	+	Antiserum 1	-
Antigen 2	+	Antiserum 2	+
Antigen 1	+	Antiserum 2	-
Antigen 3	+	Antiserum 3	+
Antigen 1 (or any except 3)	+	Antiserum 3	-

It is important that no agglutination occurs among heterologous mixtures.

Antisera can be kept in the refrigerator (when not in use) for 5 days.

Antigens made with rabbit sera should be prepared fresh daily.

Merthiolate (Thimerosol) is a preservative and antiseptic used to prevent contamination of the antisera.

All antigens and antisera are tested in this manner.

Precautions

1. The diluted sera should not be kept longer than 5 days.

2. The 0.85 NaCl + merthiolate solution shouldn't be kept longer than 5-7 days unless it is frozen.

3. Unused NaCl and merthiolate solution may be frozen. All unused reagents should be refrigerated.

4. Note which sera gives weak reaction with antigen control; i.e. 2,3 give poor reactions when testing with these sera.

   Make sure you observe the sera mixture quite closely from the time you place the drops on the tile for at least one minute.

5. Keep tile gently swirling at all times.

APPENDIX - J

MEDIA PREPARATION

Acetamide

Arabinose (see CS method)

Arginine

Bile esculin

(Rabbit) blood agar

1% Carbohydrate solutions (CS)

Catalase reagent

Crystal Violet Reagent

Gelatin

Grams Iodine

Gram Reagent

Lactose (see C.S. method)

Litmus milk

Magnesium chloride

Mannitol (see C.S. method)

m-CP2

m-E

Melibiose (see C.S. method)

m-Enterococcus

m-PA

MRS agar

m-Tec

0.85 NaCl and merthiolate solution

Oxidase reagent

Phosphate buffer, stock solution

Phosphate buffer, dilution blanks

Pyruvate broth

Saline

Skim milk agar

6.5% Sodium chloride

Sorbose (see C.S. method)

Starch agar plates

Todd Hewitt broth

TPY

Urease reagent

YN17

Medium - Acetamide Agar Slants

Ingredients:

NaCl	5.0g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.4g
Acetamide	10.0g
KH_2PO_4	0.7g
$Mg\ SO_4.7H_2O$	1.0g
Phenol Red	0.012g
Difco Bacto-Agar*	15.0g
Distilled water	1000mL

Preparation:

- * Add all ingredients except agar
  Adjust pH to 6.8; then add agar
- Heat to 92° C
  Autoclave media, empty screw cap tubes, screw caps and syringe at 121° C for 15 minutes
- Before dispensing under laminar flow, adjust pH to 6.8 ± 0.2
  Slant tubes to dry

Reading:

- The acetamide test is used for Pseudomonas aeruginosa identification. The slant is streaked along the surface and incubated for 3 days at 35° C. A bright pink is indicative of a positive reaction

Medium - Arginine Dihydrolase Medium (Thornley)

Ingredients:

Bacto Peptone (Difco)	1.0g
NaCl	5.0g
K_2HPO_4	0.3g
L-Arginine Hydrochloride	10.0g
Phenol Red	0.01 g
Bacto Agar (Difco)	3.0g
Distilled Water	1000mL

Preparation:

- Mix ingredients into Distilled water
- Adjust pH to 6.8 if necessary
- Heat to 90°C, to dissolve
- Dispense into tubes (5ml/tube)
- Autoclave 15 min., 15 psi (121° C)

Inoculation:

- Using a needle pick up growth from plate and stab straight down through centre of arginine medium. Layer top of Arginine medium with approximately 1 cm sterile paraffin oil.

Incubation:

- 35° C, up to 5 days

Reading:

- positive, medium changes from orangey-pink to bright pink at any time up to 5 days

- negative, no change

Medium - Bile Esculin Agar

Ingredients:

(Difco) Peptone	5.0g
(Difco) Beef extract	3.0g
(Difco) Oxgall	40.0g
(Sigma) Esculin	1.0g
Ferric citrate	0.5g
(Difco) Agar	15.0g
Distilled water	1000 mL

Preparation:

- Mix ingredients into Distilled water
- Heat to 90^{o} C, to dissolve
- Dispense into tubes with screw caps (5 ml/tube)
- Autoclave 15 min., 15 psi (121^{o} C)
- Cool tubes in slanted position

Inoculation:

- Transfer growth from plate and smear, with loop, over slant

Incubation:
- 35^{o} C, up to 72 hr. (3 days)

Reading:

- Positive, blackening of one-half or more of slant at any time up to 72 hr.

- Negative, blackening of less than one-half or no blackening of medium at 72 hr.

Medium - Blood Agar (Rabbit) (5%)

Ingredients:

(Difco) Blood Agar base (powder) (BAB)	40.0g
Rabbit Blood (Defibrinated)	50 mL
Distilled Water	1000 mL

Preparation:

- Mix BAB powder into Distilled Water
- Heat to 90^{o} C, to dissolve
- Autoclave 15 min. 15 psi (121^{o} C)
- Cool to approx. $50-55^{o}$ C
- Aseptically add 50 mL defibrinated rabbit blood
- Pour plates under laminar flow hood

Inoculation:

- 1. Pour plate + aerobic incubation
  2. Streak plate + anaerobic incubation

Incubation:

- Suggest, 35^{o} C streak, anaerobic, 48 hours

Reading:

- Three Haemolytic reactions possible

  1. Beta (B) - Complete clearing around colony
  2. Alpha (α) - Incomplete clearing around colony with red cells left intact close to colony
  3. Gamma (τ) - No Haemolysis

Rabbit blood is used for hemolysis testing because, even though Streptococci can produce a positive result on other animal bloods, rabbit blood helps to differentiate between the fecal streptococci since only *S. Faecalis zymogenes* produces B haemolysis.

Medium - 1% Carbohydrate Solution

Ingredients:

(Difco) Heart Infusion Broth (powder)	25.0g
Carbohydrate Sol$^{\underline{n}}$ (10% Aqueous)	100 mL
Distilled Water	900 mL
Brom Cresol Purple Solution (BCP)	1.0 mL

Preparation:

1. Stock indicator Solution
   1.6g BCP in 100 mL 95% ETOH

2. Carbohydrate Solution

Carbohydrate	10.0g
Distilled Water	100 mL

- Mix HIB powder into 900 mL Distilled Water
- Add Carbohydrate Sol$^{\underline{n}}$ (100 mL)
- Add BCP indicator (1 mL)
- Dispense into tubes (5 mL/tube)
- Autoclave 10 min, 15 psi (121° C)

Inoculation:

- Using a needle inoculate broth tube

Incubation:
- 35° C, up 72 hr. (3 days)

Reading:

- Positive, colour change from purple to yellow

- Negative, no change

The above recipe can be followed to make the following sugar solutions: lactose, mannitol.

Sugars such as arabinose, sorbose, melibiose are made slightly differently.

Make the HIB solution with indicator.

Autoclave it with test tubes and caps (separately) and syringe. Filter sterilize the carbohydrate solution (2) into the HIB after it has cooled. Syringe the solution into the tubes under the laminar flow.

The media is tested with a positive and negative to check it is working properly.

The tubes are inoculated with some culture and checked every day for 5 days for positive results and recorded. After incubation time, the result is recorded as negative if media remains purple.

Medium - Catalase Solution 3% Reagent

Ingredients:

H_2O_2 (hydrogen peroxide) 30%	5 mL
Distilled Water	45 mL

Preparation:

Keep refrigerated for maximum 2 weeks. Check before use, to make sure it works properly.

Reading:

- Drops of this reagent are placed on a microscopic slide. The solution is firstly tested with known cultures which would produce positive (bubbles) and negative (no bubbles) results when the culture is added (no mixing necessary) to the reagent.

Medium - Crystal Violet Reagent

Ingredients:

A	Crystal violet	2.0g
	Ethanol, 95%	20 mL
B	Ammonium Oxalate in 80 mL distilled water	0.8g

Preparation:

- Add A to B
- Filter

Medium - Gelatin (12%)

Ingredients:

(Difco) Heart Infusion Broth (powder)	25.0g
(Difco) Gelatin	120.0g
Distilled Water	1000 mL

Preparation:

- Mix ingredients into distilled water

NB - Add Gelatin very slowly so as not to cause clumping

- Heat to dissolve (90° C)
- Dispense into tubes (5 mL/tube)
- Autoclave 15 min, 15 psi (121° C)

Inoculation:

- Using a needle stab inoculate through Medium

Incubation:

- 35° C, for 5 days

Reading:

- After incubation remove all tubes to refrigerator (-10° C), cool until uninoculated control tube is solid when inverted. (15 min. in refrigerator)

- Positive, medium remains liquid when inverted after cooling

- Negative, medium remains solid when inverted after cooling

Medium - Gram's Iodine

Ingredients:

Iodine Crystals	1.0g
Potassium Iodide	2.0g
Distilled Water	300 mL

Preparation:

- Dissolve chemicals in a small amount of distilled water. Make up to 300 mL.

Medium - Gram Reagent, 3% KOH

Ingredients:

Potassium Hydroxide	1.5g
Distilled Water	50 mL

Application:

- This reagent should be kept maximum 2 weeks refrigerated and always tested prior to use.
- The reagent should first be tested with (2) controls which would give a positive and negative result to make sure it is working properly.
- Drops of the reagent are placed on a microscope slide.
- A loop of culture is mixed with the reagent for approximately 60 seconds.

Reading:

- A Gram positive result (G+) occurs when your mixture does not thicken or become stringy when you lift the loop from the mixture.

- A Gram negative culture when mixed with KOH becomes syrupy or mucoid. When the loop is raised from the mixture it adheres and becomes stringy.

Medium - Litmus Milk

Ingredients:

(Difco) Litmus Milk Powder	100.0g
Distilled Water	1000 mL

Preparation:

- Weigh litmus milk powder and add it to the distilled water. Put it on hot plate just to dissolve.
- Dispense 5 mL per test tube.
- Autoclave for 15 min, 121°C.

The litmus milk test was performed on all fecal streptococci isolates. The culture was inoculated into the tube and incubated at 37^{O} C and checked for up to 7 days.

There are many reactions which can occur in litmus milk (which is a mauve, when uninoculated)

Reduction -	The solution or a part of such will turn straw-like (beige)
Acid Curd -	Reduction usually occurs in acid curds. Plus there is a solid curd, pellet on the bottom of the tube which does not move. There, also, may be a clear whey-like liquid on the top.
Alkaline Curd -	Reduction usually occurs in alkali curds as well. There is a much softer, pliable curd on the bottom which can move or run. And on the top, there is a red to maroon liquid, opaque phase.
Proteinization -	Sometimes, what was once an alkali curd can become all liquid (may be same colour and texture as the top phase in an alkali curd).

Medium - Stock Magnesium Chloride Solution

Ingredients:

$MgCl_2$	38.0g
Distilled Water	1000 mL

Preparation:

- Dissolve $MgCl_2$ in the distilled water.
- Keep refrigerated.

This solution is used to make buffered dilution blanks (along with using stock Phosphate Buffer solution).

Use 1.25 mL stock Phosphate Buffer and 5 mL Stock Magnesium chloride solution per litre of distilled water.

Dispense into bottles and autoclave 15 min, 15 lb.

Medium - m-CP2

Ingredients:

(Difco) SFP Agar Base	52.5g
L-cysteine Hydrochloride	0.5g
Distilled Water	1000 mL

Preparation:

- Dissolve at boiling temperature
- Autoclave at 121° C for 15 min.
- Cool to 50° C and then filter sterilize (separately):

sodium resazurin	2mg
Neomycin	0.15g
Sodium Azide	0.2g
indoxyl-B-D-glucoside	0.6g

Reading:

- Target colonies (i.e. Clostridium perfringens) are yellow with a black centre which can extend to circumference and are not surrounded by a blue halo.

Medium - (Dufour's Modified) m-E

Ingredients:

Peptone	10g
Yeast Extract	30g
Sodium Chloride	15g
Sodium Azide	0.15g
Actidione (= cycloheximide store at 4° C)	0.05g
Agar, Bacto (Difco)	15g
Distilled Water	1000 mL

Preparation:

- Heat to 90° C.
- Autoclave for 15 min., 121° C.
- After autoclaving, cool the media to 55-60° C
- Add asceptically. Add all separately:

	for 1000 mL
Nalidixic Acid	240 mg in 3 ml sterile distilled water and 0.2 ml 10N NaOH, mix and add to medium
Indoxyl-B-D-glucoside	500 mg in 5 ml ethanol (95%). Mix well; then add 5 ml distilled water and add to medium
Triphenyltetrazolium chloride (TTC)	20 mg add just before pouring.

Add very last

Final pH 7.1 ± 0.1 (adjust to 7.1, if necessary)
Pour media under a laminar flow into square petri dishes

This media is used to enumerate and isolate fecal streptococci.

Membrane filtration is performed on a sample. The filters are placed on the media (which, because of its very high salt concentration makes it very harsh on the colonies). The plates are incubated inverted at 41.5°° C for 48 hr. in a cakette, moistened with paper towelling.

Target colonies appear as a pink button surrounded by a blue halo.

Medium - m-Enterococcus Agar

Ingredients:

(Difco) Bacto m-Enterococcus Agar	42.0g
Distilled Water	1000 mL

Preparation:

- *Sterilize spatulas and distilled water with magnetic stirrer for 15 min. at 121° C
- Allow to cool
- Weigh out m-Enterococcus agar. Flame beaker before adding media to water
- Sterilize a thermometer by immersing it in ethanol and wiping it dry. Place inside media
- Heat media to 90° C
- Once media reaches 90° C, change over to another (cold) hot plate. Quick cool the media by placing it in a cakette with ice. Keep changing the ice to bring the temperature down rapidly.

- When the media has reached 55-60° C, check the pH, adjust (if necessary) to 7.2 ± 0.2. Pour the media under a laminar flow hood into square petri dishes.

This media is used to enumerate and isolate fecal streptococci. The media is placed inverted in a cakette which has been moistened with paper towelling. Plates were incubated at 37°° C for 48 hours. Target colonies may be pink to maroon, and of any size. The plates with approximately 8-150 colonies were counted. With the presence of 2,3,5 - Triphenyl-Tetrazolium chloride (TTC) all streptococci appear as pink, maroon, along with background or undesired colonies.

Without using TTC strep. faecium and strep. faecalis variants all appear pink, maroon. However strep. faecium casseliflavus appears yellow.

Medium - MOE Formula mPA Agar (Sterile Technique)

Use sterile glassware

L-Lysine Monohydrochloride	5.0g
Yeast Extract	2.0g
Xylose	2.5g
Sodium Thiosulphate	5.0g
Magnesium Sulphate, Anhydrous	1.5g
Sucrose	1.25g
Lactose	1.25g
Sodium Chloride	5.0g
Ferric Ammonium Citrate (green)	0.80g
Sodium Desoxycholate	0.10g
Phenol Red	0.08g
Distilled Water, Sterilized	800 mL (to make 1 litre)
(Difco) *Agar Bacto	15.0g

*Adjust pH to 7.60. Then add agar.

Preparation:

- Heat to 93° C until boiling starts to dissolve agar.
- DO NOT AUTOCLAVE
- Cool to 60° C. Add antibiotics and mix thoroughly

- Antibiotics are dissolved in 200 mL sterile distilled water

Antibiotics	Amount per litre
Sulfapyridine	0.176g
Kanamycin sulphate	0.0085g
Nalidixic acid	0.037g
Actidione (Cycloheximide)	0.150g

- Heat antibiotic mixture to approximately 50° C (no higher) to help dissolve (if necessary).
- Check surface pH, adjust, if necessary, to 7.1
- Cool to 50° C (no higher than 60° C)
- Pour into petri dishes
- Final pH 7.1 ± 0.1

This media is used for enumerating *Pseudomonas aeruginosa* count.
Moistened paper towelling is added to a cakette, inverted plates are placed inside and incubated at 41.5° C for 48 hr. Target colonies may appear brown, black. Tan colonies from the filter are streaked on Skim Milk Agar for *Ps. aeruginosa* confirmation.

Medium - MRS Agar Plates

Ingredients:

*Cysteine Hydrochloride	0.3g
Gibco MRS Broth	52.0g
Difco Agar, Bacto	15.0g
Distilled Water	1000 mL

Preparation:

- Dissolve 52.0g MRS broth into 1000 mL distilled water.
- Add 15 gm agar. Heat to 90° C to dissolve.
- Autoclave for 15 min. at 121° C.
- After autoclaving, cool media to 55-60° C.

*Asceptically, add cysteine hydrochloride.

- Pour media under a laminar flow hood into round petri dishes 100 mm x 15mm.

This media was used to isolate, cultivate Bifidobacteria either by spread plating or membrane filtration.
Plates were incubated anaerobically in an anaerobic jar using BBL GAS Pack anaerobic systems (70304) with BBL Gas Pack indicator strips (70504) for 24-48 hr. at 37° C.
Colonies appeared white, round; opaque and mucoid.

Medium - m-TEC Agar (Thermotolerant *E. coli*)

Ingredients:

Proteose Peptone No. 3	5.0g	Difco
Yeast Extract	3.0g	Difco
Lactose	10.0g	BDH
Sodium Chloride	7.5g	BDH
Potassium Phosphate, Dibasic K_2HPO_4	3.3g	Fisher
Potassium Phosphate, Monobasic, KH_2PO_4	1.0g	Fisher
Sodium Lauryl Sulphate	0.2g	BDH
Sodium Deoxycholate	0.1g	BDH
Bromocresol Purple	0.08g	Difco
Bromo Phenol Red	0.08g	BDH
Difco Agar, Bacto	15.0g	Difco
Distilled Water	1000 mL	

Preparation:

- Mix all ingredients into 1000 mL distilled water.
- Heat to 90° C to dissolve the agar.
- Autoclave 15 min./15 P.S.I./121° C.
- Allow to cool to 50° C after autoclaving and pour, asceptically into sterile plates.
- Final surface pH should equal 7.1 ± 0.1.

Because m-Tec media is the least selective of the media used, a negative control (passing sterile phosphate buffer through unit) was placed on media before beginning each new sample. This ensured the unit and buffer were sterile.

This media is used for enumerating fecal coliforms and E. coli.

Moistened paper towelling is added to a cakette, inverted plates are balanced in the centre and a jar, frozen with 50 mL tap water is placed at either end of cakette. Cakette is incubated at 44.5° C for 23 ± 1 hr.

Yellow-yellow green colonies are counted from a filter with 10-150 colonies present.

Medium - 0.85% NaCl and Merthiolate Solution

Ingredients:

Merthiolate (refrigerated)	0.01gm
NaCl	.85gm
Distilled Water	100 mL

Preparation:

- Dissolve merthiolate, NaCl in distilled water.
- Autoclave for 15 min., 121° C.
- Refrigerate, when cooled.

This solution is used as a diluting reagent for Pseudomonas Antibody Sera.

To dilute sera (i.e. antisera) 0.9 mL of the above solution is mixed with 0.1 mL sera (i.e. antisera).

To prepare Pseudomonas aeruginosa isolates for serotyping, after a pure culture is grown in saline overnight, it is autoclaved and centrifuged. The above solution (0.75 mL) is added to the pellet and mixed. And this solution is used for tge slide agglutination test.

Medium - Oxidase Reagent

NNN^1N^1 - Tetramethyl - p - Phenylenediamine	0.25g
Ascorbic Acid	0.025g
Sterile Distilled Water (warm) not higher than 35° C	25 mL

Preparation:

- NEVER STIR - GENTLY SHAKE (I.E. ROTATE)

- * Add ascorbic acid to distilled water to give a 0.1% solution.
- Add 0.25g of NNN^{1}N^1 - Tetramethyl - p - Phenylediamine dihydrochloride
- Store in dark, 30 mL eye dropper bottles at 4° C.
- Reagent should last 7-14 days, but should not be used if it turns blue.

The oxidase reagent drops are applied to a Whatman No. 1 filter paper which has been cut to fit a petri dish. The oxidase test is performed once the paper has dried. The reagent is firstly tested with cultures which would produce a positive result, and a negative result. A wooden applicator is used to pick up some culture and pressed on the oxidase paper. A positive is indicated if the spot turns blue (purple); while a negative appears as no change.

Medium - Phosphate Buffer Stock Solution

Ingredients:

Potassium dihydrogen phosphate (KH_2PO_4)	34.0g
Distilled Water	volume up to 1 L.

Preparation:

1. Dissolve KH_2PO_4 in 500 mL distilled water (in 2 L. beaker)
2. Adjust pH to 7.2 ± 0.2 with I.ON NaOH.
3. Pour this amount into 1 L. graduated cylinder and make up to 1 L. volume.
4. Pour the solution back in to the same beaker and autoclave it at 121° C for 15 min.

After autoclaving, when cooled, pour the sterilized solution in to a sterilized 1 L. volumetric flask.

May be kept at 4° C for no longer than 1 month.

The above stock solution is used to make:

a) Wash waters for membrane filtration (MF)
Use 1.25 mL stock solution per 1 L. distilled water
Autoclave 15 min., 121° C.
Refrigerate.

b) Dilution blanks for samples for MF or spread plating. Use 1.25 mL stock phosphate buffer solution and 5 mL stock magnesium chloride per L. distilled water. Fill dilution blanks with approximately 104 mL/bottles (99 mL + 4 mL for evaporation). Autoclave for 15 min. at 121° C. Refrigerate.

Medium - Pyruvate Broth

Ingredients:

Tryptone	10.0g
Yeast Extract	5.0g
K_2HPO_4	5.0g
NaCl	5.0g
Pyruvic acid, sodium salt	10.0g
Bromothymol blue (BTB)	0.04g
Distilled Water	1000 mL

Preparation:

- Mix all ingredients except BTB into distilled water.

- * BTB solution
  Mix 0.5g bromothymol blue in 5 mL IN NaOH.
  Add 0.4 mL of this solution into basal medium.
- Adjust pH to 7.2
- Dispense 5 mL per tube
- Autoclave 15 min. at 121° C

The streptococccus culture is inoculated into pyruvate broth. The test tube is inoculated for 24 hours (only) at 35° C. A lime green or yellow represents a positive result.

Medium - Saline (physiological, 0.85%)

Ingredients:

Sodium chloride	8.5g
Distilled Water	1000 mL

Preparation:

- Dissolve salt in the distilled water.
- If required in test tubes, dispense before autoclaving.
- Autoclave at 121° C, 15 lb.
- Keep refrigerated.

Medium - Skim Milk Agar

Ingredients:

Difco Skim Milk Powder	100g per litre
Difco Bacto-Agar	15.0g
Distilled Water	1000 mL

Preparation:

- Slowly add skim milk powder to 500 mL distilled water and stir without heat for 30 min.
- In a separate beaker, slowly add the agar to 500 mL distilled water while stirring. Heat the agar solution slowly to 90-92° C (10-12 min.)*
- Autoclave the two solutions separately for 12 min. at 121° C. After autoclaving, cool the 2 solutions to approximately 55° C then [at which time, add the skim milk solution asceptically to the agar solution. Stir the mixed solution for an additional 2-3 min. (Temperature should be 50-52° C)].
- Dispense asceptically into sterile plates [20 mL in round (100 x 13 mm) plates].
- Prepare a small plate for quality control pH reading.
- Adjust to 6.4 ± 0.2 at 25° C (surface reading).

This media is used to determine many characteristics associated with Pseudomonas aeruginosa, such as pigmentation, caseinase production, fluorescence and oxidase reaction. Plates are streaked for isolated colonies and incubated for 48 hr. at 37° C.

Pseudomonas aeruginosa usually appears yellow-green, lime green, dark green, brown on Skim milk agar.

A clearing of the media indicates caseinase production. Under ultra-violet light source, Pseudomonas aeruginosa colonies fluorese. Using the plate, provided the sample appears pure, it can be tested with the oxidase reagent (Ps. aeruginosa +) (Please see oxidase Reagent for detailed information).

Medium - 6.5% Sodium chloride

Ingredients:

(Difco) Heart Infusion Broth (powder)	25.0g
NaCL	65.0g
Distilled Water	1000 mL

Preparation:

- Mix ingredients into Distilled Water
- Dispense into tubes (5mL/tube)
- Autoclave 15 mi, P.S.I. (121° C)

Inoculation:

- Using a needle pick some growth from the plate and inoculate lightly into the broth - no visible turbidity should be detected after inoculation.

Incubation:

- 35° C, up to 72 hr. (3 days)

Reading:

- Positive, growth (visible turbidity) at any time up to 3 days

- Negative, no visible turbidity at 3 days

Medium - Starch Agar Plates

Ingredients:

(Difco) Blood Agar Base powder	40.0g
Soluble starch	20.0g
Distilled Water	1000 mL

Preparation:

- Slowly, add 20g soluble starch to 1000 mL distilled water.
- Add 40g Blood Agar Base to the mixture. Heat the solution to 90° C to dissolve.
- Autoclave 15 min., 121° C.
- Pour in a laminar flow hood into round petri dishes 100 mm x 15 mm.
- Refrigerate plates.

The fecal streptococci isolates were tested for starch hydrolysis by placing a 1 line streak on a 6 sector plate. The plates were incubated at 37° C for 5 days. At which time they were flooded with Gram's Iodine solution and checked after 30 min. A clearing of the media indicates a positive starch result.

Medium - Todd-Hewitt Broth

Ingredients:

(Difco) Todd Hewitt Broth Powder	30.0g
Distilled Water	1000 mL

Preparation:

- Mix powder into distilled water
- Dispense into tubes (5 mL/tube)
- Autoclave, 15 min., 15 psi (121° C).

Inoculation:

- For testing growth at 10° C and 45° C using a needle, transfer some growth from the plate into the broth - No visible turbidity should be detected after inoculation.

Incubation:

- 10° C up to 5 days
- 45° C

Reading:

- 10° C and 45° C
- Positive, - visible growth in broth
- Negative, - clear non turbid broth

Medium - TPY

Ingredients:

BBL Trypticase	10.0g
BBL Phytone	5.0g
Glucose	5.0g
Difco Yeast Extract	2.5g
Tween 80	1 mL
Cysteine hydrochloride	0.5g
K_2HPO_4	2.0g
$MgCl_2.6H_2O$	0.5g
$ZnSO_4.7H_2O$	0.25g
$CaCl_2$	0.15g
$FeCl_3$	trace
Difco Bacto, Agar	15.0g
Distilled Water	1000 mL

Preparation:

- Final pH is 6.5 after autoclaving at 121° C for 25 min.
- The media was poured under a laminar flow hood into round petri dishes 100 mm x 15 mm.

This media was used to isolate, cultivate Bifidobacteria either by spread plating or membrane filtration.

Plates were incubated anaerobically in an anaerobic jar using BBL Gas Pack anaerobic systems (70304) with BBL Gas Pack indicator strips (70504) for 48 hr. at 37° C.

Medium - Urease Reagent

Ingredients:

Urea	10.0g
Phenol Red Solution	0.5 mL
1.0g of phenol red in 10 mL ethanol (0.25 in 2.5 mL)	
Distilled Water	500 mL

Preparation:

- Weigh the phenol red in a weigh boat. Add the alcohol and mix to dissolve the dye.
- Weigh the urea and add it to the distilled water.
- Add 0.5 mL of the prepared phenol red solution to the urea solution.
- Adjust pH to 5.0 ± 0.2

- Store in a pyrex container for up to 2 weeks in the refrigerator.
- Check (and adjust) pH before use.

The urease test is performed to determine the presence of E. coli.

After the m-Tec (media used to enumerate Fecal coliforms) is counted, the filter is placed on a pad, contained in a small petri dish, soaked with urease reagent (but not dripping). The filter is left for 15 min. and recounted. Yellow-yellow green colonies are indicative of E. coli.

Medium - YN17(b) (MF media for Bifidobacteria)

Ingredients:

Yeast extract	20.0g
Polypeptone	10.0g
Lactose	10.0g
Casamino acid	8.0g
NaCl	3.2g
Bromocresol green	0.30g
Cysteine hydrochloride	0.40g
(Difco) Bacto-agar	15.0g
Distilled Water	1000 mL

Preparation:

- Stir on medium heat until agar dissolves.
- Autoclave for 15 min. at 121° C.
- Adjust pH to 6.9 ± 0.1.
- Cool to 60° C. and add:

Nalidixic acid	0.03g
Kanamycin sulphate	0.05g
Polymixin B sulphate	0.0062g

Target colonies on YN17 (b) appear dark blue and black.

*YN17 growth media

Follow above recipe. However, do not include bromocresol green or antibiotics.